Campylobacter jejuni and the Guillain-Barré syndrome

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ω	ohms
λ	lambda
σ	sigma
3'	three prime
5'	five prime
°C	degree Celsius
µF	micro Farads (capacitance)
µm	micrometer
µg	microgram
µl	microliter
bp	base pairs
BSA	bovine serum albumin
CO_2	carbon dioxide
Da	Dalton
DNA	deoxyribonucleic acid (cDNA-copy DNA or complementary DNA)
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
dUTP	deoxyribouridine triphosphate
et al	et ali, and others
GBS	Guillain-Barré syndrome
h	hour
HCl	hydrochloric acid
kb	kilo base pairs
kV	kilo volts
LOS	lipooligosaccharide
LPS	lipopoligosaccharide

mg	milligram
ml	milliliter
mM	millimole
M	molarity
min	minute
N_2	nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
O_2	oxygen
OD	optical density
pH	potential of hydrogen
RNA	ribonucleic acid (mRNA–messenger RNA)
RNase	ribonuclease
s	seconds
TEMED	N,N,N,N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) amino methane
Tris-HCl	tris hydrochloride
U	units
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume

LIST OF PUBLICATIONS AND PRESENTATIONS

List of publications

1. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. 2006. Exchange of lipooligosaccharide genes creates potential Gullain-Barré syndrome-inducing strains of *Campylobacter jejuni*. Infection and Immunity. 74:1368-1372.
2. **Vongsavanh Phongsisay** and Benjamin N. Fry. Bidirectional transcription of lipooligosaccharide synthesis genes from *Campylobacter jejuni*. Submitted for publication.
3. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. Expression of the *htrB* gene is essential for responsiveness of *Salmonella typhimurium* and *Campylobacter jejuni* to harsh environments. Microbiology, in press.
4. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. Evaluation of eight RNA isolation methods for transcriptional analysis in *Campylobacter jejuni*. Journal of Microbiological Methods, in press.

List of presentations

Three poster presentations (1, 2, and 3 below) and two oral presentations (2 and 3) at the 13th international workshop on *Campylobacter*, *Helicobacter*, and related organisms (www.chro2005.com), Gold Coast, Queensland, Australia, 5-8 September, 2005.

1. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. Exchange of lipooligosaccharide genes creates potential Gullain-Barré syndrome-inducing strains of *Campylobacter jejuni*.
2. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. Transcriptional analysis of the lipooligosaccharide synthesis gene cluster from the Guillain-Barré syndrome-inducing *Campylobacter jejuni* HB 93-13.

3. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. The essential and virulent *wlaNA* gene of *Campylobacter jejuni*.

Four posters presented (1 to 4 below) at the 11th Asian Conference on Diarrhoeal Diseases and Nutrition (www. ascodd2006.net), Bangkok, Thailand, March 8-10, 2006.

1. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. Exchange of lipooligosaccharide genes creates potential Gullain-Barré syndrome-inducing strains of *Campylobacter jejuni*.
2. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. Transcriptional analysis of the lipooligosaccharide synthesis gene cluster from the Guillain-Barré syndrome-inducing *Campylobacter jejuni* HB 93-13.
3. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. The essential and virulent *wlaNA* gene of *Campylobacter jejuni*.
4. **Vongsavanh Phongsisay**, Viraj N. Perera, and Benjamin N. Fry. Evaluation of eight RNA isolation methods for transcriptional analysis of the lipooligosaccharide synthesis gene cluster from *Campylobacter jejuni*.

SUMMARY

Campylobacter jejuni is an enteric bacterium that causes human gastroenteritis worldwide. Some *C. jejuni* strains exhibiting human ganglioside-like lipooligosaccharide (LOS) structures, such as GM1 ganglioside, can induce an autoimmune neuropathy of the peripheral nervous system known as the Guillain-Barré syndrome (GBS). This GBS-inducible determinant is encoded by a gene cluster, which shows a high degree of variation among *C. jejuni* strains. The experiments presented in this thesis were conducted to give a better insight into the LOS synthesis genes in relation to the pathophysiology of *C. jejuni*. Firstly, a *C. jejuni* strain without GM1-like molecules was shown to be able to take up large DNA fragments, including LOS synthesis genes, from a strain expressing GM1-like molecules and consequently be transformed into a number of potential GBS-inducible transformants, which exhibited a high degree of genetic and phenotypic diversity. The ability of *C. jejuni* to take up and integrate foreign DNA explains the genome plasticity observed in this pathogen. Secondly, while attempting to analyse transcription of the LOS gene cluster, neither published methods nor any commercially available kits for RNA isolation could produce DNA-free RNA from *C. jejuni*. Combinations of these methods were trialled and only the combination of RNAzolB, TURBO DNase treatment, and acid phenol extraction was able to produce DNA-free RNA. The RNA isolated from most *C. jejuni* strains showed different RNA patterns to that of other bacteria. In addition the RNA from *C. jejuni* seemed closely associated with DNA compared to RNA from other organisms. This might be caused by species-specific DNA conformation or chromatin structure. Thirdly, bidirectional transcription was observed in the LOS gene cluster. Both DNA strands were transcribed but transcription of the non-coding strands was at a lower rate, and both sense and antisense transcripts of each LOS gene tested were responsive to acid stress. This unusual transcription might have a potential effect on the expression of the GBS-inducing determinant. Finally, one of the LOS genes, the *htrB* gene, was further analysed. It was shown that expression of the

htrB gene affects morphology, viability, growth ability, and sensitivity to stress environments. These results showed that the LOS molecule of *C. jejuni* is involved in many processes and is an important molecule for survival.

GENERAL INTRODUCTION

1. General characteristics of *Campylobacter jejuni*

The word *Campylobacter* is derived from the Greek word "*campylos*" which means curve and the word "*bactron"* which means rod. *C. jejuni* is a microaerophilic and capnophilic bacterium, which can appear as curved rods, spiral rods, straight rods, and/or coccus forms (pleomorphism). It is motile by means of a single unsheathed polar flagellum at one or both ends. Motility appears as a unique darting and cork-screw-like movement. *C. jejuni* is mainly found in the intestinal tracts of animals, such as poultry and swine (77).

The genome sequence of *C. jejuni* strain 11168 has been recently established (81). It has a circular chromosome of 1,641,481 bp (30.6% G + C), which is predicted to encode 1,654 proteins and 54 stable RNA species. Unusually, the genome has no insertion sequences or phage-associated sequences and very few repeat sequences. One of the most striking features of the genome is the presence of hypervariable sequences. In addition, the short homopolymeric runs of nucleotides are commonly found in genes encoding the biosynthesis or modification of surface structures, or in closely linked genes of unknown function. Whole genome comparative analysis among *C. jejuni* strains suggests that genetic diversity is a major trait of this bacterium (18).

2. Diseases caused by *C. jejuni*

2.1. Campylobacteriosis

C. jejuni causes gastroenteritis (campylobacteriosis) in humans. It is predominantly characterised by inflammatory diarrhoea, abdominal pain, and/or fever. Infection of humans mainly occurs by consumption of contaminated poultry products, milk, and water (77).

2.2. Guillain-Barré syndrome

Guillain-Barré syndrome (GBS) is an autoimmune neuropathy of the peripheral nervous

system that is the most serious disease complication following bacterial and viral infections. Causative organisms include *C. jejuni*, cytomegalovirus, Epstein-Barr virus, and *Mycoplasma pneumonia*. GBS is characterised by weakness, usually symmetrical, evolving over a period of several days or more. Affected persons rapidly develop weakness of the limbs, weakness of the respiratory muscles, and areflexia (loss of reflexes) (72, 116).

GBS consists of at least 4 subtypes of acute peripheral neuropathy; acute inflammatory demyelinating polyradiculoneuropathy (AIDP), acute motor axonal neuropathy (AMAN), acute motor and sensory axonal neuropathy (AMSAN), and Fisher's syndrome (FS) (42). AIDP subtype resembles experimental autoimmune neuritis, and there is evidence for the involvement of antibodies and complement. AMAN and AMSAN subtypes are caused by antibodies to gangliosides on the axolemma that target macrophages to invade the axon at the node of Ranvier. About a quarter of patients with GBS have had a recent *C. jejuni* infection, and axonal forms of the disease are especially common in these people. The FS subtype is especially associated with antibodies to GQ1b, which damages the motor nerve terminal *in vitro* by a complement-mediated mechanism.

3. Prevalence of campylobacteriosis and Guillain-Barré syndrome

Children under five years old are most susceptible to *Campylobacter* infections (16). Among this group, the rate of illness caused by *C. jejuni* is higher than that caused by *Vibrio* spp., *Salmonella* spp., *Shigella*., pathogenic *Escherichia coli*, *Aeromonas* spp., and *Plesiomonas* spp. [M.Sc. thesis (V. Phongsisay), Faculty of Tropical Medicine, Mahidol University, Thailand].

The prevalence of GBS is fairly uniform at between one and four cases per 100,000 throughout the world, and men are about 1.5 times more likely to be affected than women (72, 103).

4. Treatment of campylobacteriosis and Guillain-Barré syndrome

In general, *Campylobacter* enteritis does not warrant antimicrobial therapy. Although campylobacteriosis is a self-limiting disease, treatment can decrease duration and severity of the illness as well as potentially prevent its complicating disease, GBS. Antibiotic treatment is necessary for patients who are acutely ill with enteritis, have persistent fever, and bloody diarrhoea. When antimicrobial therapy is indicated, erythromycin is the preferred drug for treatment. Although the incidence of GBS is rare, special care is needed for GBS patients to prevent and manage the potentially fatal complications of the disease. Plasma exchange and intravenous immunoglobulin are the treatment strategies for GBS patients (72, 112).

5. Pathogenesis of campylobacteriosis

Presently, the pathogenesis of campylobacteriosis is not fully understood; however, the disease development requires the resistance of *C. jejuni* to stress environments in the human gastrointestinal tract such as acid, bile, and osmotic pressure. In addition, the interaction between host factors and bacterial virulence factors is a crucial process for disease development. As the same *C. jejuni* isolates are observed in both diarrhoeal patients and healthy persons (13), host factors are expected to play an important role in disease development. However, its role in relation to campylobacteriosis has not yet been characterised. Furthermore, a number of antigenic determinants/bacterial components are known to contribute to the virulence of *C. jejuni*. These include CadF (52), JlpA (43), PEB1 (83), CiaB (53), plasmid (8), flagella (107), cytotoxin (56), capsule (9), and LOS (24).

5.1. CadF

The *cadF* gene encodes a protein of 326 amino acids with a calculated molecular mass of 37 kDa (CadF) (52). CadF is a conserved outer membrane protein of *C. jejuni* that specifically binds to the fibronectin of the extracellular matrix of epithelial cells. The deduced amino acid sequence exhibits 52% similarity and 28% identity to the root adhesin protein from

Pseudomonas fluorescens. Mutagenesis and *in vivo* experiments have shown that the *C. jejuni* CadF protein appears to be required for the colonisation of newly hatched leghorn chickens (119).

5.2. CiaB

C. jejuni secretes a set of proteins termed the *Campylobacter* invasion antigens (Cia proteins). These proteins are secreted from the flagellar export apparatus and are required for the internalisation of *C. jejuni* into cultured mammalian cells. For example, the *ciaB* gene that is designated the *Campylobacter* invasion antigen B encodes a protein of 610 amino acids with a calculated molecular mass of 73 kDa (CiaB). The deduced amino acid sequence of the CiaB protein shares similarity with type III secretion proteins associated with the invasion of host cells from other bacterial pathogens. Mutagenesis and *in vitro* experiments have shown that the *C. jejuni ciaB* gene is required for the secretion process and the efficient entry of this bacterium into a host cell (53, 54).

5.3. JlpA

The *jlpA* gene, a 1,116 bp open reading frame, is a species-specific lipoprotein of *C. jejuni*. It encodes a polypeptide (JlpA) of 372 amino acid residues with a molecular mass of 42.3 kDa. JlpA contains a typical signal peptide and lipoprotein processing site at the N-terminus. JlpA is a lipoprotein that is loosely associated with the cell surface. Mutagenesis and *in vitro* experiments have shown that JlpA is a *C. jejuni* adhesin involved in adherence (43).

5.4. Peb1

Peb1 is a *C. jejuni* adhesion protein that is a homolog of cluster 3 binding proteins of bacterial ABC transporters and is identical to the previously identified cell-binding factor 1, CBF1, in *C. jejuni*. Peb1 is encoded by the 0.78 kb *peb1A* locus encoding a 259-residue polypeptide with a molecular mass of 28 kDa. Mutagenesis experiments have shown that the *C. jejuni*

peb1A locus plays an important role in epithelial cell interactions and in intestinal colonisation in a mouse model (82, 83).

5.5. Plasmids

A number of *C. jejuni* isolates have been shown to possess plasmids between 30 and 40 kb in size (87). The *C. jejuni* strain 81-176 harbours two plasmids (pTet and pVir), each approximately 35 kb in size (8). The first plasmid, pTet, carries the *tetO* gene. Transfer of the *tetO* gene among *C. jejuni* strains has been demonstrated to occur both *in vivo* (chicken) and *in vitro* (7, 87). Since chicken products are frequently reported as the infectious source of *C. jejuni* (77), the rapid and spontaneous transfer of the *tetO* gene potentially contributes to the high prevalence of tetracycline resistance in both *Campylobacter* strains isolated from chickens and humans. The second plasmid, pVir, contains four open reading frames (ORFs). ORF1 through ORF3 encode proteins that display significant identity to the products of the *comB1*, *comB2*, and *comB3* genes, respectively, of *H. pylori* P1, which are involved in DNA uptake via natural transformation in *H. pylori* (41). Similarly, mutation of the plasmid gene, *comB3*, in *C. jejuni* 81-176 causes reduced adherence and invasion of INT407 cells and a reduced natural transformation frequency (8). ORF4 encodes a protein whose highest identity match is to the *H. pylori* protein JHP1316/HP1421. This *H. pylori* protein is a member of a paralogous family that has significant identity to VirB11 of the *Agrobacterium* type IV secretion system (96, 106). The *H. pylori* VirB11-like paralogous family (HP0525 and HP1421) has been proposed to function in a type IV secretion system required for virulence (17). In *C. jejuni* 81-176, mutation of a *virB11* homolog has resulted in reduced adherence and invasion of INT407 cells and reduced virulence in the ferret diarrhoeal disease model (8).

5.6. Flagella

Flagella-mediated motility is recognised to be one of the major factors contributing to virulence in *C. jejuni*. *C. jejuni* has two almost identical flagellin genes, which encode the structural subunit of the flagellum. The two flagellin genes, *flaA* and *flaB*, each 1.7 kb, are

located adjacent to one another, but they have different promoters (36, 75). The *flaB* gene is not needed for motility, while the *flaA* gene is essential for motility (107), and plays a role in colonisation (111).

5.7. Cytotoxin

C. jejuni has been demonstrated to possess a cytolethal distending toxin (CDT) that causes cells to arrest in the G (2)/M transition phase of the cell cycle (56). CDT activity requires the function of three genes: *cdtA*, *cdtB*, and *cdtC*. It has been proposed that CDT is a tripartite toxin consisting of CdtB as the enzymatically active subunit, with CdtA and CdtC as the heterodimeric B subunit required for the delivery of CdtB.

5.8. Capsule

Capsular polysaccharides (CPSs) are found on the surface of a large number of bacterial species. CPSs are known to play an important role in bacterial survival and persistence in the environment and often contribute to pathogenesis. The recent genomic sequencing of *C. jejuni* NCTC 11168 has revealed the presence of a cluster of genes encoding proteins with sequence similarity to proteins involved in the transport of type II/III capsules (81). Analysis of capsule synthesis gene clusters from different *C. jejuni* strains shows a high degree of variation among strains, ranging from 15 to 34 kb (47). Comparison of the determined CPS sequences of the HS:1, HS:19 and HS:41 strains with the sequenced strain, NCTC11168 (HS:2), provides evidence for multiple mechanisms of structural variation including exchange of capsular genes and entire clusters by horizontal transfer, gene duplication, deletion, fusion, and contingency gene variation. In contrast, the HS:23, HS:36 and HS:23/36 CPS sequences were highly conserved (47). Karlyshev *et al.* (48) have shown that site-specific insertional mutagenesis of *kpsM*, *kpsS* or *kpsC* (capsule synthesis genes) in several strains results in the loss of a high-molecular-weight glycan (capsule) (48). Moreover, Karlyshev and colleagues (48) have also demonstrated that *kps* mutants in five distinct serogroups of *C. jejuni* lost the ability to be typed in the Penner scheme (84), indicating that capsule, rather than

lipooligosaccharide, as previously thought, is the serodeterminant molecule. Furthermore, the capsule is involved in the virulence of *C. jejuni* since a capsule-deficient *C. jejuni* 81-176 *kpsM* mutant exhibits an increased surface hydrophobicity and serum sensitivity, a reduced ability to invade INT407 cells, and is less virulent in a ferret diarrhoeal disease model (9).

5.9. Lipooligosaccharide

The lipooligosaccharide (LOS) of *C. jejuni* is a major surface molecule. It consists of two parts, the core oligosaccharide (Fig. 1) and the lipid A (Fig. 2). The core oligosaccharide is divided into the inner core and the outer core. In some strains of *C. jejuni*, the core oligosaccharide resembles human gangliosides such as GM1, GM2, and GQ1b (Fig. 1) (3-5, 32, 62, 93). This molecular mimicry can induce GBS (117). In addition, the outer core part of the LOS molecule plays a role in the virulence of *C. jejuni* (24), whilst the lipid A of the LOS molecule possesses endotoxic properties (73).

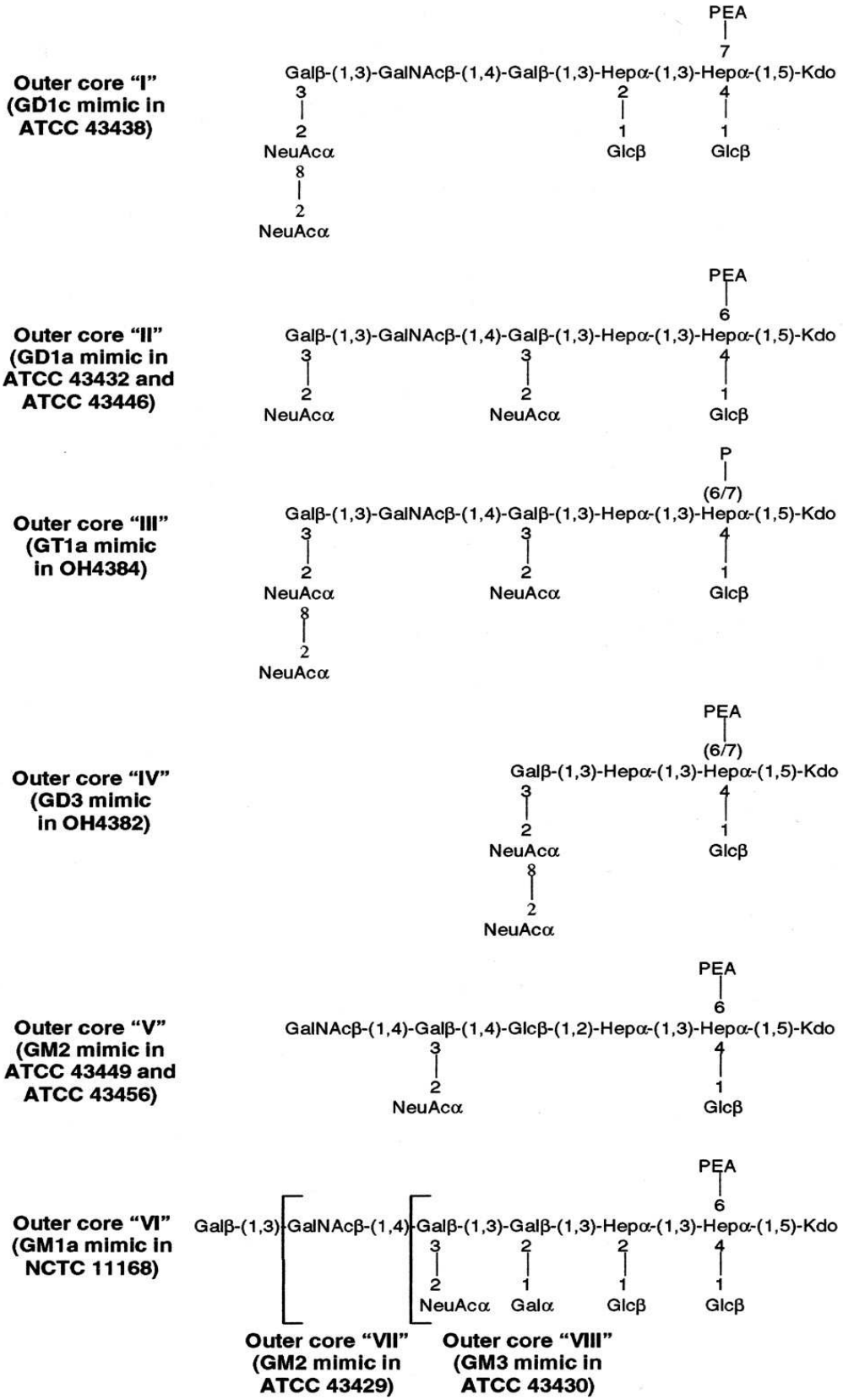

Figure 1. Outer regions of the LOS structures expressed by *C. jejuni* strains [from Gilbert *et al*. 2002 (32)].

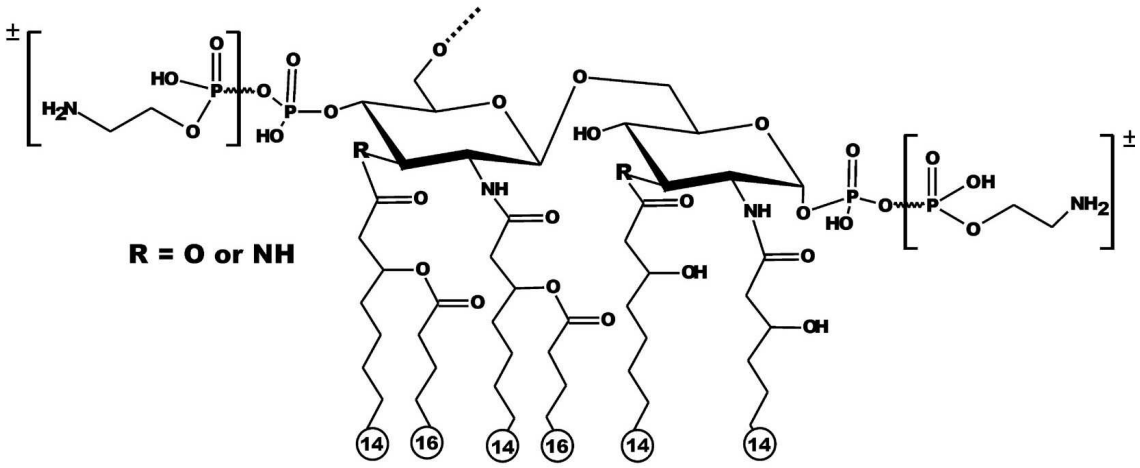

Figure 2. Lipid A structure of *C. jejuni* NCTC 11168 [from Szymanski *et al*. 2003 (102)].

6. Pathogenesis of Guillain-Barré syndrome

Presently, the pathogenesis of GBS is not fully understood; however, it is known that the molecular mimicry that exists between the LOS of some *C. jejuni* strains and the human gangliosides in nerve tissue can induce GBS. In addition, as the same *C. jejuni* isolates have observed in both GBS and non-GBS patients (112), host factors are expected to play an important role in GBS development.

7. Lipooligosaccharide synthesis gene cluster

The LOS of *C. jejuni* is encoded by two gene clusters, *wla*I and *wla*II. The *wla*I gene cluster is highly conserved in *C. jejuni* (25, 81). It is involved in both protein glycosylation and LOS synthesis (24, 25). In *C. jejuni* strain 81116, it contains 12 consecutive genes: *galE*, *wlaB*, *wlaC*, *wlaD*, *wlaE*, *wlaF*, *wlaG*, *wlaH*, *wlaI*, *wlaK*, *wlaL*, and *wlaM* (25). Their putative functions were previously described by Fry and colleagues (24, 25) and are shown in Table 1. In contrast, the *wla*II gene cluster exhibits a high degree of genetic variation among strains (32, 80) (Fig. 3, also see Table 2). Some of these genes have been experimentally characterised and found to be involved in the induction of GBS and the biosynthesis of LOS and capsule.

Table 1. Deduced proteins present in the *wla*I gene cluster of *C. jejuni* 81116 and their homologues (25)

C. jejuni protein	Similar Protein (% identity)	Organism	Putative function	Accession no.
GalE†	GalE (37.5)	*Haemophilus influenzae*	UDPglucose 4-epimerase	A64063
	GalE (36.3)	*Neisseria meningitidis*	UDPglucose 4-epimerase	S39638
WlaB	HetA (30.1)	*Anabaena sp.*	Polysaccharide involvement	P22638
	HlyB (30.1)	*Escherichia coli*	Export haemolysin	P08716
WlaC	TrsD (28.8)	*Yersinia enterocolitica*	Glycosyltransferase	S51263
	AmsD (27.4)	*Erwinia amylovora*	Glycosyltransferase	S52144
WlaD	Orf8.7 (27.2)	*Yersinia pseudotuberculosis*	Abequosyltransferase	L01777
	TrsB (23.3)	*Yersinia enterocolitica*	Glycosyltransferase	S51261
WlaE	AmsD (25.1)	*Erwinia amylovora*	Glycosyltransferase	S52144
	TrsD (24.9)	*Yersinia enterocolitica*	Glycosyltransferase	S51263
WlaF	STT-3 (19.5)	*Caenorhabtidis elegans*	Oligosaccharyltransferase	P46975
	Wzy (18.8)	*Escherichia coli*	O-antigen polymerase	P37748
WlaG	CpsF (30.8)	*Proteus mirabilis*	Glycosyltransferase	L36873
	RfbF (27.1)	*Serratia marescens*	Galactantransferase	L34167
WlaH	WbaP (39.5)	*Salmonella enterica*	Galactosyltransferase first step	P26406
	WbaP (38.5)	*Haemophilus influenzae*	Galactosyltransferase	B64099
WlaI	NeuD (28.1)	*Escherichia coli*	Polysialic acid capsule synthesis	U05248
	LpxD (26.6)	*Salmonella enterica*	Acyltransferase	P18482
WlaK‡	RfbE (32.2)	*Vibrio cholerae*	Perosamine synthetase	S28471
	DegT (29.0)	*Bacillus stearothermophilus*	Transamination	P15263
WlaL	CapD (38.5)	*Staphylococcus aureus*	Capsule synthesis	P39853
	TrsG (37.5)	*Yersinia enterocolitica*	Acetylgalactosamine synthesis	S51266
WlaM	AcfB (26.9)	*Vibrio cholerae*	Accessory colonization factor	L25660

†, Upstream of the *galE* gene is the *Cj1132c* gene encoding a hypothetical protein Cj1132c that is present in both *C. jejuni* strains 81116 and 11168; ‡, upstream of the *wlaK* gene is the *wlaJ* gene encoding a putative integral membrane protein that is present in strain 11168 but not 81116 (25, 81).

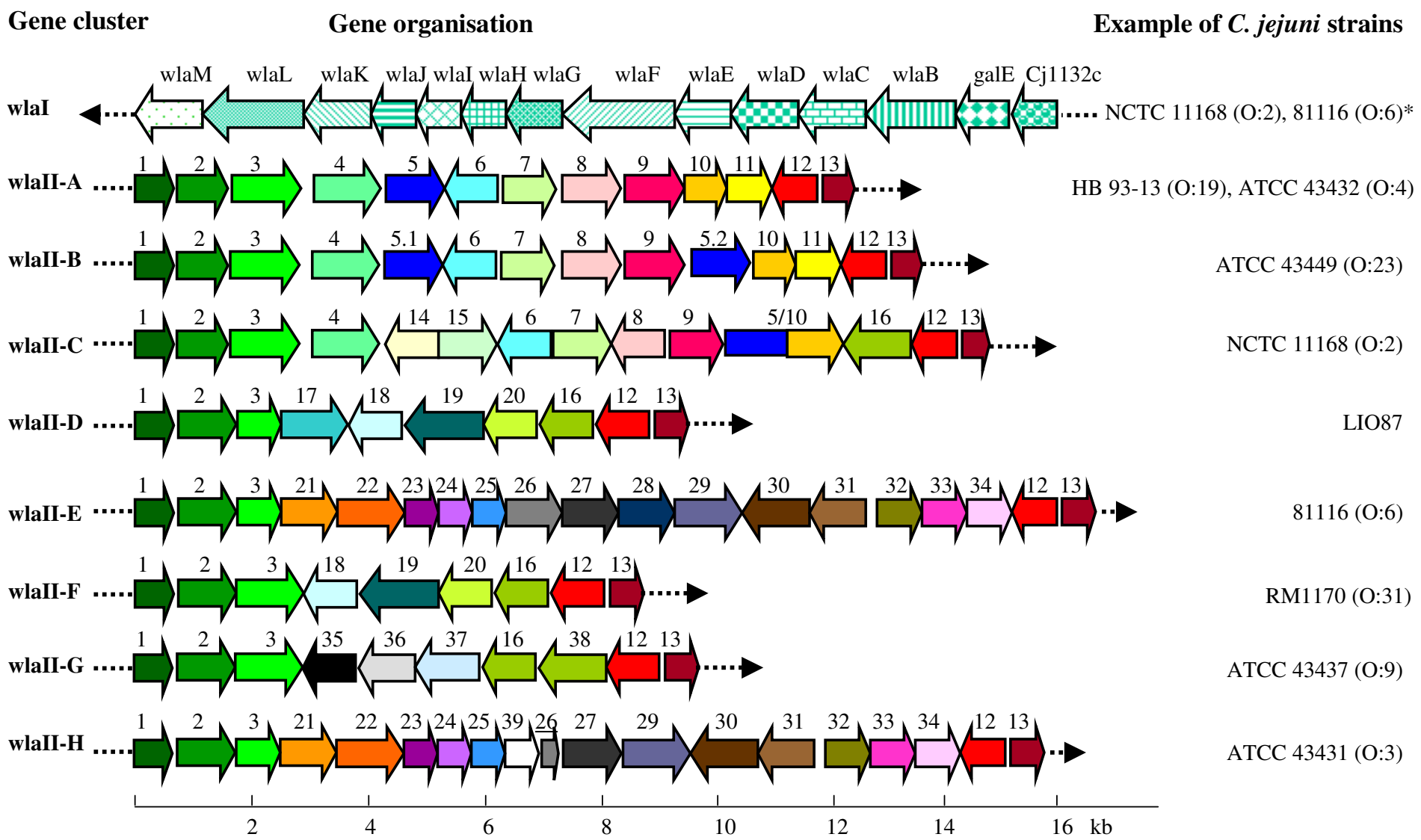

Figure 3. LOS synthesis gene clusters of *C. jejuni*. The GenBank accession numbers are AL111168 for NCTC 11168 (81), Y11648 for 81116 (25), AY297047 for HB 93-13 (74), AF215659 for ATCC 43432 (32), AF401529 for ATCC 43449 (32), AL139077 for NCTC 11168 (81), AF400669 for LIO87 (33), AF343914 and AJ131360 for 81116 (26, 76), AY434498 for RM1170 (79, 80), AY436358 for ATCC 43437 (80), AY800272 and AF411225 for ATCC 43431 (31, 80). *, strain 81116 does not possess the *wlaJ* gene (25). 1-39 are ORFs; underline, partial ORF 26.

Table 2. Functions of the genes found in the *wla*II-LOS synthesis gene clusters of *C. jejuni* strains (also see Figure 3)

ORF*	Homologous gene	Function (reference)
1	*waaC*	Heptosyltransferase I (45)
2	*htrB* (*wlaNA*)	Putative Lipid A biosynthesis acyltransferase (32, 81)
3	*wlaNC*	Putative glucosyltransferase (32, 81)
4	*wlaND*	Putative galactosyltransferase (32, 81)
5	*cgtA*	β-1, 4-*N*-Acetylgalactosaminyltransferase (29, 35)
6	*cgtB*	β-1, 3-Galactosyltransferase (29, 62)
7	*cstII*	α-2, 3 or α-2, 3/ α-2, 8-sialyltransferase (29, 35)
8	*neuB* (*neuB1*)	Sialic acid synthase (63)
9	*neuC* (*neuC1*)	*N*-Acetylglucosamine-6-phosphate 2-epimerase (35)
10	*neuA* (*neuA1*)	CMP-Neu5Ac synthetase (32)
11	*wlaVA*	Putative acetyltransferase (32, 81)
12	*wlaQA* (*waaV*)	Putative glycosyltransferase (32, 81)
13	*waaF*	Heptosyltransferase II (76)
14	*Cj1137c*	Putative glycosyltransferase (32, 81)
15	*Cj1138*	Putative glycosyltransferase (32, 81)
16	*Cj1145c*	Hypothetical protein (32, 81)

*, Only the ORFs (1-16) are summarised accoding to the available data from the genome sequence of *C. jejuni* NCTC 11168 (81) and the publications described in the text.

7.1. *galE*

The *C. jejuni galE* gene is located in the *wla*I gene cluster (25). It encodes a UDP-glucose 4-epimerase, which catalyses the interconversion of UDP-galactose and UDP-glucose involved in the synthesis of the LOS core region (24). Mutation of the *galE* gene in *C. jejuni* results in truncated LOS, reduced adherence and invasion of INT407 cells, and reduced natural transformation frequency, indicating that the *galE* gene is involved in virulence. However, the *C. jejuni galE* mutant is still able to colonise chickens to the same level as the parent strain, and the serum resistance and hemolytic activity of this mutant does not change compared to the parent strain (24). In addition, *in vivo* experiments have shown that the *galE* gene is essential for the formation of human ganglioside-like LOS structure (94), which can induce GBS (117).

7.2. *waaC*

The *C. jejuni waaC* gene is located in the *wla*II gene cluster. Kanipes *et al.* (45) have experimentally shown that this gene encodes a heptosyltransferase I that catalyses the transfer of the first L-glycero-D-manno-heptose residue to a 3-deoxy-D-manno-octulosonic residue in the (Kdo)-lipid A (45). Mutagenesis experiments have demonstrated that the *C. jejuni waaC* gene contributes to the synthesis of both LOS inner core and capsule carbohydrate (45).

7.3. *cstII*, *cgtA*, *cgtB*, *neuC*, and *neuA*

Phase variation of the *C. jejuni cgtB* gene affects the ganglioside-like LOS structure (62). The *cgtB* homologous gene in *C. jejuni* NCTC 11168 encodes a β-1, 3 galactosyltransferase, which is responsible for converting GM2-like to GM1-like LOS structures. It is essential for the formation of human ganglioside-like LOS structure. In *C. jejuni* NCTC 11168, the presence of an intragenic homopolymeric tract (poly G) renders the expression of a functional *cgtB* gene product phase variable, resulting in distinct cell populations with alternate GM1 or

GM2 ganglioside-mimicking LOS structures. Similarly, phase variation of the *cgtB* homologous gene is also observed in *C. jejuni* NCTC 12500.

Phase variation in the LOS-synthesis genes of *C. jejuni* affects not only LOS structure but also its invasion capacity (37). It has been observed that a population of cells of *C. jejuni* strain 81-176 produces a mixture of LOS cores, which exhibit primarily structures mimicking GM2 and GM3 gangliosides, with few structures mimicking GD1b and GD2. These multiple LOS structures are due to the presence of a homopolymeric tract of G residues within the *cgtA* gene (orf 5.2), a putative β-1, 4-N-acetylgalactosaminyltransferase (GalNAc), which affects the length of the ORF resulting in a change in LOS structure from GM2 to GM3 ganglioside like structure. Site-specific mutation of the *cgtA* gene in 81-176 resulted in a major LOS core structure lacking GalNAc that exhibits GM3 ganglioside and increased invasion of INT407 cells. In comparison, site-specific mutation of the *neuC1* gene resulted in the loss of sialic acid in the LOS core and a reduced resistance to normal human serum, but it had no affect on invasion of INT407 cells.

Sialylation of the LOS cores affects immunogenicity and serum resistance of *C. jejuni* strain MSC57360 (35). The *cstII* gene encodes a sialyl transferase and the *neuC* gene (*neuC1*) encodes an N-acetylmannosamine synthetase that is part of the biosynthetic pathway of N-acetylneuraminic acid (NeuNAc). The *C. jejuni* strain MSC57360 lacking the *cstII* or *neuC* gene exhibits identical phenotypes. The LOS cores of these mutants exhibit an increased electrophoretic mobility, the loss of reactivity with cholera toxin (CT), and an enhanced immunoreactivity with a hyperimmune polyclonal antiserum generated against whole cells of *C. jejuni* MSC57360. In comparison, mutation of the *cgtA* gene [orf 5, fused with orf10 (*neuA1*), Fig. 3] resulted in LOS cores intermediate in electrophoretic mobility between that of the parent and the mutants lacking NeuNAc, the loss of reactivity with CT, and a reduced immunoreactivity with hyperimmune antiserum. In addition, a mutant with a nonsialylated LOS core is more sensitive to the bactericidal effects of human sera than the parent or the

mutant lacking GalNAc (35). Interestingly, mutation of the *cgtA* gene resulted in the loss of GalNAc but not NeuNAc from the LOS core (35). Possibly, the fusion protein has either lost CMP-NeuNAc synthetase activity or there are additional copies of genes encoding enzymes with the same function (35). Indeed, NCTC 11168, in addition to containing *Cj1143* (*neuA1*), contains two other copies of *neuA* alleles, *Cj1311* (*neuA2*) and *Cj1331* (*neuA3* or *ptmB*). The *neuA3* or *ptmB* allele has been shown to be involved in posttranslational modification of flagellin of *Campylobacter coli* VC167 (34), but the role of this gene in LOS biosynthesis in VC167, whose core is uncharacterised, has not yet been elucidated.

7.4. *neuB*

The *C. jejuni neuB* gene is involved in the synthesis of both LOS and flagella (63). In *C. jejuni* NCTC 11168, there are three N-acetyl neuraminic acid (NANA) synthetase genes termed *neuB1* (*neuB*, Table 2), *neuB2*, and *neuB3*. The *neuB1* gene is mapped in the *wla*II gene cluster, while the others are located outside the LOS gene cluster. Mutation of the *neuB1* gene affects LOS core structure, while mutation of the *neuB2* and *neuB3* genes does not affect LOS, but the *neuB3* mutant is aflagellate and non-motile. No phenotype change is evident for *neuB2* mutants in strain NCTC 11168, but for strain G1 the flagellin protein from the *neuB2* mutant shows an apparent reduction in molecular size relative to the wild type.

7.5. *waaF*

The *C. jejuni waaF* gene is located in the *wla*II gene cluster. It encodes a heptosyltransferase II, which catalyses the transfer of the second L-glycero-D-manno-heptose residue to the core oligosaccharide moiety of the LOS molecule. Mutation of the *waaF* gene in *C. jejuni* resulted in the production of a truncated core oligosaccharide, the failure to bind specific ligands, and the loss of serum reactive GM1, asialo-GM1, and GM2 ganglioside epitopes. In contrast to the *waaC* gene, mutation of the *waaF* gene does not affect the higher-molecular-weight polysaccharide supporting the production of a LOS-independent capsular polysaccharide by *C. jejuni* (76).

SCOPE OF THE PRESENT STUDY

This thesis describes the characterisation of the LOS synthesis gene cluster in relation to the pathophysiology of *C. jejuni* as follows:

Chapter I: Exchange of LOS synthesis gene clusters and adjacent regions between non-GBS and GBS-inducing *C. jejuni* strains.

Chapter II: Evaluation of current RNA isolation methods for transcriptional analysis of the *wla*II-LOS synthesis gene cluster from *C. jejuni* strains.

Chapter III: Transcription analysis of the *wla*II-LOS synthesis gene cluster from the GBS-inducing *C. jejuni* strain HB 93-13.

Chapter IV: Physiological examination of the pleiotropic functions of the LOS-synthesis *htrB* gene of *C. jejuni* HB 93-13.

CHAPTER I

Exchange of LOS synthesis gene clusters and adjacent regions between non-GBS and GBS-inducing *C. jejuni* strains

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ABSTRACT

Human ganglioside-like structures, such as GM1, found on some *Campylobacter jejuni* strains have been linked to inducing the Guillain-Barré syndrome (GBS). This study investigated interstrain exchange of the *C. jejuni* LOS synthesis genes via natural transformation. A *C. jejuni* strain 81116 (GM1 negative) was incubated with genomic DNA isolated from the *C. jejuni* O:4 *wlaVA* mutant (GM1 positive and kanamycin resistant). One hundred and fifty kanamycin resistant transformants were randomly picked and immunologically probed with the cholera toxin B subunit (CTB), which is GM1 specific. Surprisingly, 145 of the 150 transformants reacted with CTB while only 5 transformants were CTB-negative. PCR-RFLP analysis showed that the host strain 81116 acquired various DNA fragments (up to 45 kb), including LOS synthesis genes, from donor DNA using various integration points that mostly located outside the LOS gene cluster. Consequently, strain 81116 transformed into a number of GM1-expressing transformants, which exhibited a high degree of variation in their LOS gene clusters and adjacent regions. Moreover, PFGE analysis revealed that horizontal exchange of large DNA fragments (up to 388 kb) occurred throughout the genome of strain 81116. Furthermore, silver staining of LOS isolated from the transformants after SDS-PAGE showed various LOS patterns and most of them were different from the LOS pattern of parent strain 81116. Therefore, these results showed that a *C. jejuni* strain without GM1-like molecules can transform into a number of potential GBS-inducible strains, which exhibit a high degree of genetic and phenotypic diversity, following natural transformation.

INTRODUCTION

The Guillain-Barré syndrome (GBS) is a postinfectious autoimmune neuropathy that can occur following campylobacteriosis. Affected persons rapidly develop weakness of the limbs, weakness of the respiratory muscles, and areflexia (72). Presently, the pathogenesis of GBS is not fully understood. However, since similar *C. jejuni* strains have been isolated from both GBS and non-GBS patients (112), host factors also play an important role in GBS development. In addition, a study by Yuki *et al*. (117) showed that rabbits, which had been sensitised with *C. jejuni* lipooligosaccharide (LOS), developed anti ganglioside-GM1 immunoglobulin G (IgG) antibodies and flaccid limb weakness (117). Paralysed rabbits had pathological changes in their peripheral nerves that were identical to those present in GBS (117). Moreover, immunisation of mice with *C. jejuni* LOS generated a monoclonal antibody (MAb) that reacted with GM1 and bound to human peripheral nerves. The MAb and anti-GM1 IgG from GBS patients blocked muscle action potentials in a muscle-spinal cord coculture (117). These results indicated that anti-GM1 antibodies can cause muscle weakness (117), and the molecular mimicry that exists between the human gangliosides, including GM1, and the *C. jejuni* LOS is one of the GBS-inducible determinants.

The *C. jejuni* LOS is partly encoded by the *wla*II gene cluster that has been shown to exhibit a high degree of variation among strains (32, 80). Presently, the function of individual LOS genes is not fully understood; however, the *wlaND*, *cgtA*, *cgtB*, *cstII*, *neuB*, *neuC*, *neuA*, and *waaF* genes are essential for the formation of human ganglioside-like LOS structures which can induce GBS (35, 37, 62, 63, 76, 117). Upstream of the *waaC* gene, the *wla*I gene cluster is found which is highly conserved in *C. jejuni* strains (25, 81). The *wla*I locus is mainly involved in protein glycosylation, although at least one gene, *galE*, is also involved in LOS synthesis (24).

Natural transformation is the ability of a bacterium to take up genetic material, which can be integrated into the chromosomal DNA via homologous recombination. Exchange of LOS

synthesis genes was recently shown for a *C. jejuni* strain isolated from a patient with GBS (30). This study hypothesised that a non-GM1 strain could take up LOS synthesis genes *in vitro* and potentially become a GBS-inducible strain.

MATERIALS AND METHODS

Bacterial growth

C. jejuni was grown on Columbia agar plates supplemented with 5% defribinated horse blood under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37ºC for 16 h. *E. coli* was grown in Luria-Bertani (LB) broth or agar at 37˚C for 16 h. Media were supplemented with kanamycin (50 μg/ml) when appropriate.

Preparation of whole cell lysates

Bacteria were grown as previously described. Bacterial cells were harvested in 1 ml of PBS and centrifuged at 3,300 × g (6,000 rpm) for 6 min. The pellet was resuspended in 2 ml of PBS and then homogenised on ice at 70% amplitude for at least 3 times for 20 s (each) with 20 s interval using a Branson Digital Sonifier. The protein concentration was determined using a modified Lowry procedure (14).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used for analysis of individual proteins/components presented in bacterial whole cell lysates. A separating gel (12%) was prepared by mixing the following: 4.78 ml of MilliQ water, 3.75 ml of 1.5 M Tris-HCl (pH 8.8), 6 ml of 30% stock acrylamide solution, 150 μl of 10% (w/v) SDS. Gel polymerisation was initiated by adding 50 μl of 10% ammonium persulfate in MilliQ water (freshly prepared) and 7.5 μl of TEMED. The gel was poured gently into the space formed between two glass plates to the level of 1-2 cm below the upper edges of the plates. The gel was overlaid with MilliQ water and allowed to polymerise for at least 30 min without disturbance. The stacking gel (4%) was prepared in the same manner as described in the separating gel. It consisted of 3 ml of MilliQ water, 1.25 ml of 0.5 M Tris-HCl (pH 6.8), 0.65 ml of a 30% acrylamide solution, 100 μl of 10% (w/v) SDS, 50 μl of 10% ammonium persulphate (freshly prepared), and 10 μl of TEMED. Water and unpolymerised acrylamide were removed from the separating gel. The stacking gel was

poured on top of the polymerised separating gel. The comb was inserted and then the stacking gel was allowed to polymerise for at least 30 min. The comb was then carefully removed and the wells were rinsed with 1 × electrophoresis buffer (5 × buffer: 12.12 g of Tris base, 57.16 g of glycine, 4 g of SDS, 800 ml of MolliQ water, pH 8.3). The whole assembly was placed in an electrophoresis chamber, and the chamber was filled with 1 × electrophoresis buffer. Whole cell lysate was diluted 1:2 in sample buffer (1 ml of 0.5M Tris-HCl (pH 6.8), 2 ml of glycerol, 1.6 ml of 10% SDS (w/v), 0.2 ml of 0.5% bromophenol blue (w/v), 0.4 ml of 2-6-mercaptoethanol, and 2.8 ml of MilliQ water), heated at 100˚C for 4 min, and loaded (appropriate volume) into wells in the gel. The SeeBlue^{TM} Plus2 Pre-Stain standard (Invitrogen) was used as the molecular weight marker. Electrophoresis was carried out at 100 V until the tracking dyes reached the separating gel edge. After electrophoresis, the gel was removed and processed for further applications.

Electrophoretic transfer

Bacterial components that had been separated on the gel were electrically transferred onto nitrocellulose membrane. The separating gel was soaked in 1 × transfer buffer (12.12 g of Tris base, 57.60 g of glycine, 3,200 ml of MilliQ water, and 800 ml of methanol) to equilibrate for 5 min. A piece of nitrocellulose membrane, eight pieces of 3M whatman paper (filter paper), and scotch brite pads were also soaked in transfer buffer for 5 min. The transfer cassette was made in the following order: 1 soaked scotch brite pad, 4 sheets of whatman 3M paper, polyacrylamide gel, membrane, 4 soaked sheets of filter paper, and scotch brite pad on the back section of the cassette. The cassette was closed and submerged in a trans-blot tank with the membrane on the anode side of the gel. The tank was filled with transfer buffer and the electrophoretic transfer was carried out at 70 V for 2 h.

Immunoblotting with cholera toxin B subunit (CTB)

The regions of nitrocellulose membrane surrounding the bound proteins or LOSs were blocked by incubation with blocking solution (3% BSA and 0.5% gelatin in 0.01M PBS, pH

7.4) for 1 h on a rotary shaker at room temperature. The blocking solution was removed and the membrane was washed for 3 times for 15 min (each) by gentle shaking in PBS-T (0.05% Tween-20 in 0.01M PBS, pH 7.4). The membrane was incubated with 25 ml of CTB solution [0.4 µg/ml CTB (Calbiochem) in dilution buffer (0.2% BSA and 0.2% gelatin in 0.01M PBS, pH 7.4)] at room temperature for 1 h with gentle shaking and the membrane was then washed 3 times with excess volume of PBS-T as previously described. The membrane was placed in a solution of goat anti-CTB (1:10,000 in dilution buffer) for 1 h at room temperature with gentle shaking and washed as mentioned above. After washing, the membrane was placed in a solution of rabbit anti-goat HRP (diluted 1:3,000 in dilution buffer, Bio-Rad) for 1 h at room temperature with gentle shaking and washed as previously described. The membrane was incubated for 10 min in 50 ml of 1/15M phosphate buffer (pH 7.6). Finally, the membrane was placed in a plastic box containing a freshly prepared substrate solution (1 ml of 4-chloro-1-naphthol in methanol (30 mg/ml), 10 ml of methanol, 49 ml of 1/15 PB, and 30 µl of H_2O_2) with gentle shaking until the red-brown bands appeared. The enzyme-substrate reaction was stopped by rinsing the membrane with distilled water and the membrane was air dried.

Construction of the *C. jejuni wlaVA* mutant

The mutated *wlaVA* construct was kindly provided by Dr. Viraj N. Perera (School of Applied Sciences, RMIT University, Australia). This construct consists of a pBluescript plasmid carrying the *wlaVA* gene of *C. jejuni* strain HB 93-13, which is inactivated by a kanamycin resistance cassette (Km). It was introduced into *C. jejuni* strains using natural transformation and/or electro-transformation.

Natural transformation

C. jejuni was transformed with DNA using natural transformation as previously described (108). *C. jejuni* was grown as previously mentioned. Bacterial cells were harvested in 1 ml of heart infusion broth. Bacterial suspension (50 µl) was transferred into a microcentrifuge tube

containing 1 ml of heart infusion agar. The lid was closed and pierced. The tube was incubated under microaerobic conditions at 37˚C for 3 h. After incubation, 10 μg of plasmid or chromosomal DNA was added and the mixture was incubated at 37˚C for another 3 h. The mixture containing cells and DNA was then transferred onto blood agar plates supplemented with appropriate antibiotics, and the plates were incubated under microaerobic conditions at 37˚C for at least 2 days.

Preparation of *C. jejuni* competent cells

Competent cells were prepared by treating *C. jejuni* cells with SG solution containing 9% sucrose and 15% glycerol in MilliQ water. This solution was sterilized by filtration through a 0.2-μm membrane. *C. jejuni* was grown as previously described. Bacterial cells were washed once with 1 ml of cold SG solution at 3,300 × g for 6 min at 4˚C. After centrifugation, the pellet was resuspended in 0.5 ml of cold SG solution, and 40 μl of bacterial suspension (competent cells) was aliquoted into microcentrifuge tubes and chilled on ice for immediate use or stored at -70˚C for long term storage.

Introduction of DNA into competent *C. jejuni* by electrotransformation

Competent cells (40 μl) were mixed with genomic DNA or plasmid DNA (0.1-1 μg) and the mixture was chilled on ice for 1 min. If the competent cells had been stored at -70˚C, they were first thawed on ice for 30 min before mixing with DNA. The entire mixture was transferred into a chilled electroporation cuvette (0.2 cm width, Bio-Rad). The Gene Pulser apparatus (Bio-Rad) was set at 25 μF, 2.48 kV, and 200 Ω. The mixture was pulsed once at these settings and the cuvette was immediately filled with 1 ml of SOC medium (91). The mixture was transferred to Mueller Hinton agar plates or blood agar plates without antibiotic supplements and incubated under microaerobic conditions at 37˚C overnight. Bacterial cells were harvested in 0.2 ml of heart infusion broth and grown on blood agar plates supplemented with appropriate antibiotics under microaerobic conditions at 37˚C for at least 2 days.

PCR-RFLP of the entire *wla*II gene cluster

Amplification of the entire *wla*II gene cluster was carried out by using the Expand Long Template PCR system (Roche). All steps were performed on ice. Using the genome sequence of *C. jejuni* NCTC 11168 (81), the primer WaaC-F, which is located in the *waaC* gene (5'-CCGTGGTTTTGCAATTTATC-3', nucleotide 53 to 72), and the primer WaaF-R, which is located in the *waaF* gene (5'-AAGTTCTTGTTCGGCTTTTC-3', nucleotide 594 to 575), were designed. The reaction mixture was prepared in a total volume of 50 µl containing 1 µl of DNA template (100 ng/µl), 5 µl of buffer 3, 1.75 µl of dNTP mix (10 mM each, Roche), 2 µl of primer WaaC-F (50 ng/µl), 2 µl of primer WaaF-R (50 ng/µl), 0.75 µl of enzyme mix, and 37.5 µl of MilliQ water. The PCR conditions were programmed on a GeneAmp PCR system 2400 (Perkin Elmer) as follows: 1 cycle of 94˚C for 2 min; 10 cycles of 94°C for 10 s, 47˚C for 30 s, 68˚C for 12 min; 20 cycles of 94˚C for 15 s, 47°C for 30 s, 68˚C for 12 min (add 20 s in each successive cycle); and 1 cycle of 68˚C for 7 min. After amplification, the PCR product was digested with 40 U of *Hind*III (Promega) at 37˚C overnight according to the manufacturer's instruction. After digestion, 20 µl of digestion product mixed with 2 µl of 11 × loading buffer (91) was loaded on a 1.5% (w/v) agarose gel in 1 × TAE buffer (91). A standard marker of lambda DNA digested with *Pst*I was used. Electrophoresis was conducted at 100 V for 90 min in 1 × TAE buffer. The gel was stained in ethidium bromide solution (6 µg/ml) for 10 min, destained in running water for 40 min, and photographed using a gel documentation system (GelDoc, Bio-Rad).

PCR-RFLP of the entire *wla*I gene cluster

Amplification of the entire *wla*I gene cluster was carried out by using the Expand Long Template PCR system (Roche) as previously described in the PCR-RFLP of the *wla*II gene cluster. Using the sequence data of the *wla*I gene cluster of *C. jejuni* 81116 (25), the primer GalE1, which is located in the *galE* gene (5'-GCGGTGGTGCAGGTTATATAGG-3', nucleotide 17 to 38), and the primer WlaM, which is located in the *wlaM* gene (5'-

GCTCACTCCACCGATAAGAT-3', nucleotide 831 to 812), were designed and used for PCR. The PCR product was digested with 40 U of *Hha*I (Promega) at 37˚C overnight according to the manufacturer's instruction.

PCR-RFLP of the DNA fragment downstream of the *waaF* gene (*waaF* to *Cj1152c*)

PCR was carried out using *pfu* polymerase (Roche). Using the genome sequence of *C. jejuni* NCTC 11168 (81), the primer WaaF-F, which is located in the *waaF* gene (5'-TACATCTTCCCACCTGGTTA-3', nucleotide 14 to 33), and the primer Cj1155c-R, which is located in the *Cj1155c* gene (5'-ATGCTTGCACCATACCTTTG-3', nucleotide 116 to 97), were designed and used for PCR. The reaction mixture was prepared in a 50 μl total volume of 1 × *pfu* buffer containing 200 μM of each dATP, dTTP, dCTP, dGTP, 100 ng of primer WaaF-F, 100 ng of primer Cj1155c-R, 100 ng of DNA template, and 5 U of *pfu* polymerase. The PCR conditions were as follows: 94˚C for 1 min (initial denaturation); 35 cycles of 94˚C for 30 s, 47˚C for 1 min, 72˚C for 11 min; 72˚C for 3 min (final elongation). The PCR product was digested with 30 U of *Hind*III (Promega) at 37˚C overnight according to the manufacturer's instruction.

PCR-RFLP of the DNA fragment downstream of the *Cj1152c* gene (*Cj1152c* to *dnaX*)

PCR was carried out using *pfu* polymerase (Roche) as previously described, except that the annealing temperature was 60˚C. Using the genome sequence of *C. jejuni* NCTC 11168 (81), the primer Cj1155c-F, which is located in the *Cj1155c* gene (5'-AGGTATGGTGCAAGCATTAT-3', nucleotide 100 to 119), and the primer DnaX-R, which is located in the *dnaX* gene (5'-TAGGCTCTCCAAAACAATCT-3', nucleotide 1516 to 1497), were designed and used for PCR. The PCR product was digested with 30 U of *Hind*III (Promega) at 37˚C overnight according to the manufacturer's instruction.

Pulse-field gel electrophoresis (PFGE)

C. jejuni was grown as previously described. Bacterial cells were washed once with 1 ml of PBS at 3,300 × g for 6 min and then resuspended in 800 µl of PIV buffer (10 mM Tris-HCl, 1M NaCl, pH 7.6). Four hundred microliters of bacterial suspension (OD_{600} of 0.57-0.82) was gently mixed with 400 µl of melted low melting agarose (2% agarose in PIV buffer). The mixture was dispensed into wells of reusable plug molds and allowed to solidify at room temperature for at least 30 min. The plugs were transferred into plastic tubes (10 ml in size) containing lysis buffer [0.5 M EDTA (pH 9.0), 1% sarcosine, 1 mg/ml proteinase K] and the tubes were then incubated at 50°C with gentle shaking overnight. After incubation, lysis buffer was substituted by 2 ml of PMSF solution and the tube was then incubated at 37˚C for 1 h with gentle shaking to inactivate proteinase K. Inactivation of proteinase K was repeated once. PMSF solution was prepared by mixing 1 ml of 18.6 mg/ml PMSF in isopropanol (completely dissolved) with 25 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the mixture was incubated at 37˚C for 15 min. After inactivation of proteinase K, the PMSF solution was substituted by TE buffer and the tubes were incubated at room temperature for 30 min with gentle shaking. This step was repeated twice. The plugs were added to fresh TE buffer and stored at 4°C. The plugs were cut into small pieces (1 mm width) and the intact chromosomal DNA embedded inside the agarose was digested with 30 U of *Sac*II at 37˚C overnight according to the manufacturer's instructions. The plugs were washed once with TE buffer and stored in 0.5 × TBE buffer at 4˚C. Digested DNA fragments were separated on a 1.4% agarose gel (molecular agarose grade) at 14˚C in 0.5 × TBE buffer using the LKB gene navigator (Pharmacia). The PFGE conditions were as follows: pulse time between 0.5 s to 20 s (interpolation) and run time for 23 h.

LOS isolation

C. jejuni was grown at 37˚C for 2 days under microaerobic conditions as previously described. Bacterial cells were harvested from 2 plates and washed once with 5 ml of PBS at 5,445 × g (5,500 rpm) for 10 min. The pellet was resuspended in 2 ml of solution I (50 mM Tris-HCl, 2 mM EDTA, pH 8.5). Bacterial cells were homogenised on ice at 70% amplitude for 3 times for 30 s (each) with 20 s intervals using a Branson Digital Sonifier. Homogenised samples were centrifuged at 1,312 × g (2,700 rpm) for 20 min. The supernatants (1.5 ml) were transferred into microcentrifuge tubes and centrifuged at 16,100 × g (13,200 rpm) at 4˚C for 1 h. After the supernatants were discarded, 100 µl of solution II (2 mM Tris-HCl, pH 7.8) and 300 µl of Laemmli sample mix [2.5 ml of 0.5 M Tris-HCL (pH 6.8), 4 ml of 10% SDS, 2 ml of 100% glycerol, 1 ml of β-mercaptoethanol, 2 ml of 0.02% bromophenol blue, and 3.5 ml of water] were added to the pellets. The mixture was boiled for 15 min, and after 20 µl of proteinase K (1 mg/ml) was added, it was incubated at 56˚C for at least 1 h.

Separation of LOS components using Tricine-SDS-PAGE

LOS components were separated on maxi-gels. The assembly of the sandwich glass plates was performed as described in the instruction manual (Bio-Rad). The separating gel was prepared by mixing the following: 5.7 ml of MilliQ water, 3.9 ml of glycerol, 12.37 ml of gel buffer (3 M Tris base, 0.3% SDS, pH 8.45), 15.45 ml of 40% acrylamide stock. The mixture was gently mixed and polymerisation was initiated by adding 300 µl of 10% ammonium persulfate in MilliQ water (freshly prepared) and 30 µl of TEMED. The gel was poured gently into the space between the two glass plates to the level of 3-4 cm below the upper edges of the plates. The gel was overlaid with MilliQ water and allowed to polymerise for 2 h. The stacking gel was prepared by mixing the following: 5.55 ml of MilliQ water, 2.25 ml of gel buffer, and 1.2 ml of 40% acrylamide stock. The gel polymerisation was initiated by adding 75 µl of 10% ammonium persulfate and 7.5 µl of TEMED. The stacking gel was poured on

top of the polymerised separating gel. The comb was inserted and the stacking gel was left to polymerise for 1 h. The comb was then carefully removed and the wells were rinsed and cleaned with cathode buffer (0.1M Tris base, 0.1 M Tricine, 0.1% SDS, pH 8.25). The whole assembly was placed in an electrophoresis chamber. The chamber was filled with 300 ml of cathode buffer (inside) and 2000 ml of anode buffer (outside) (0.2 M Tris base, pH 8.9). The LOS samples (10-50 µl) were loaded into the wells in the gel. The SeeBlue^{TM} Plus2 Pre-Stain standard (Invitrogen) was used as the molecular weight marker. Electrophoresis was carried out at 130 V at 4˚C for 18 h or until the tracking dyes reached the bottom edge of the separating gel. After electrophoresis, the gel was removed and prepared for silver staining.

Silver staining of LOS components separated using Tricine-SDS-PAGE

All steps were performed at room temperature. The gel containing LOS components was fixed in fixing solution (40 % ethanol, 5 % acetic acid) overnight. The gel was then oxidised in oxidising solution (0.7 % periodic acid, 40 % ethanol, and 5 % acetic acid) for 5 min. The oxidised gel was washed with distilled water for 3 times for 15 min (each). After the water was drained from the gel, the freshly prepared silver staining reagent (28 ml of 0.1 M NaOH, 2 ml of NH_4OH, 5 ml of 20% $AgNO_3$, and 115 ml of MilliQ water) was added and the gel was incubated for 10 min with gentle shaking. The stained gel was washed with distilled water for 3 times for 15 min (each). The colour was developed by placing the stained gel in a container containing formaldehyde solution (50 mg of citric acid, 0.5 ml of 37% formaldehyde, and 1,000 ml of distilled water) for 10-30 min. The colour development was stopped by transferring the gel to a container containing stop solution (10 g of Tris base, 5 ml of 80% acetic acid, 185 ml of distilled water). The stained gels were stored in 1% glycerol in MilliQ water.

RESULTS AND DISCUSSION

Acquisition of a GM1-positive phenotype by WT 81116

In this study, we hypothesised that a non-GM1 strain could take up LOS synthesis genes *in vitro* and potentially become a GBS-inducible strain. To test this hypothesis, *C. jejuni* strain 81116 (Penner serotype O:6, named WT 81116), which was originally isolated from a human waterborne outbreak of gastroenteritis (78), was selected as the host cell in a natural transformation experiment. The LOS of this strain does not react with the CTB, which is GM1-specific. *C. jejuni* Penner serotype O:4 (named WT O:4) was selected as the donor cell because (i) it strongly reacted with CTB using Western blot analysis, (ii) the *C. jejuni* O:4 *wlaVA* mutant (named O:4 Δ*wlaVA*) could be successfully constructed by introducing the selection marker for kanamycin resistance and still reacted with CTB, and (iii) mutating the *wlaVA* gene in a strain HB 93-13 (O:19) did not affect the LOS structure, the LOS sugar composition, and the ganglioside mimicry (Viraj N. Perera, unpublished data). *C. jejuni* 81116 was transformed with chromosomal DNA of O:4 Δ*wlaVA* using natural transformation. Transformants were screened on selective medium supplemented with kanamycin. The transformation efficiency of WT 81116 was approximately 2×10^2 CFU per 10 µg of genomic DNA. One hundred and fifty kanamycin resistant colonies were randomly picked and immunologically probed with CTB to screen for GM1-positive transformants. Surprisingly, 145 of the 150 colonies reacted with CTB while only 5 colonies were CTB-negative (Fig. 1.1, selected transformants). Therefore, these results showed that *C. jejuni* 81116 could be transformed into a number of potential GBS-inducible transformants.

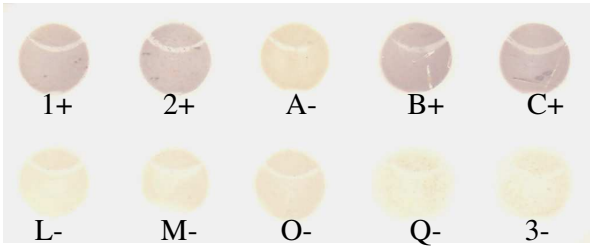

Figure 1.1. Dot blot ELISA probed with CTB. 1, WT O:4; 2, O:4 Δ*wlaVA*; 3, WT 81116. A, L, M, O, and Q are all CTB-negative transformants. B and C are representative CTB-positive transformants. +, CTB positive; –, CTB negative.

Genetic exchange of the *wla*II-LOS synthesis gene cluster

To identify which O:4 Δ*wlaVA* genes had been acquired by the CTB-positive transformants, PCR-restriction fragment length polymorphism (PCR-RFLP) with *Hind*III as the restriction enzyme was performed. The primers WaaC-F and WaaF-R were used to amplify the entire *wla*II gene cluster of WT O:4 (13.558 kb), O:4 Δ*wlaVA* (14.055 kb), WT 81116 (16.308 kb), 13 selected CTB-positive transformants (B, C, D, E, F, G, H, I, J, K, N, P, and R), and all 5 CTB-negative transformants (A, L, M, O, and Q) by PCR. For the CTB-positive transformants, the entire *wla*II gene cluster (16.308 kb) was replaced by the *wla*II locus of O:4 Δ*wlaVA* (14.095 kb) as the PCR-RFLP patterns of these transformants were identical to the pattern of O:4 Δ*wlaVA* and markedly different from that of WT 81116. For the CTB-negative transformants L, M, O, and Q, a partial exchange of LOS synthesis genes was observed. Presumably, some essential gene(s) for the synthesis of the GM1-like LOS and, hence, CTB binding is missing from their *wla*II gene clusters. Surprisingly, CTB-negative transformant A had received the complete *wla*II locus of O:4 Δ*wlaVA* (Fig. 1.2, also see Fig. 1.1). A possible explanation could be a change in the length of some of the homopolymeric tracts found in this gene cluster or mutations in the LOS synthesis genes (62) or other genes that are essential in the expression of the CTB-binding epitope. These results showed that part and even the entire *wla*II gene cluster was easily taken up and integrated in the genome of *C. jejuni* 81116, resulting in new strains carrying GM1-like LOS structures.

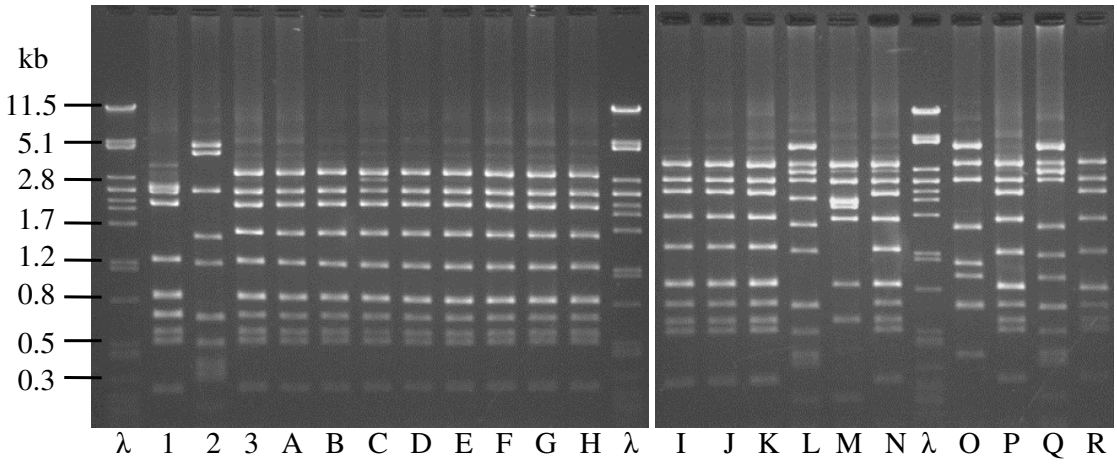

Figure 1.2. PCR-RFLP patterns of the *wla*II gene cluster digested with *Hind*III. λ, λ-DNA digested with *Pst*I; 1, WT O:4; 2, WT 81116; 3, O:4 Δ*wlaVA*; and A to R, transformants.

Identification of the integration point upstream of the *waaC* gene

To identify the integration point upstream of the *waaC* gene, *Hha*I-PCR-RFLP was performed. The primers GalE1 and WlaM were used to amplify the *wla*I gene cluster of WT 81116 (14.061 kb), 81116 transformants (A to J), WT O:4, and O:4 Δ*wlaVA*. The PCR products of approximately 14 kb were obtained for all strains. PCR-RFLP patterns of transformants showed a high degree of variation within the *wla*I gene clusters, and those were different from the patterns of WT 81116 and O:4 Δ*wlaVA* (Fig. 1.3). Restriction mapping analysis showed that WT 81116 had randomly integrated DNA fragments from the O:4 Δ*wlaVA* into its genome using various integration points. Most of them were positioned in the region of the *galE* gene as a 698-bp DNA fragment containing the partial *galE* gene was missing from the *wla*I loci of transformants A, B, C, D, F, H, I, and J. A previous study in a *C. jejuni* strain GB11 also evidenced the integration point in the *galE* region (30). Other sites were distributed throughout the *wla*I gene cluster such as in the *wlaK*, *wlaF*, *wlaE,* and *wlaC* genes.

Identification of the integration point downstream of the *waaF* gene

To identify the integration point downstream of the *waaF* gene, *Hind*III-PCR-RFLP was performed. The primers WaaF-F and Cj1155c-R were used to amplify a 5.1-kb DNA fragment ranging from the *waaF* gene to the *Cj1155c* gene by PCR. A PCR product of approximately 7 kb was obtained for the WT 81116, while a 5.1-kb PCR product was obtained for WT O:4, O:4 Δ*wlaVA*, and transformants B, C, D, E, F, G, H, and J. A PCR product could not be amplified from transformants A and I. RFLP analysis of the PCR products showed that all transformants tested had the pattern of the donor DNA, and the sites of recombination appeared to be located in or downstream of the *Cj1155c* gene.

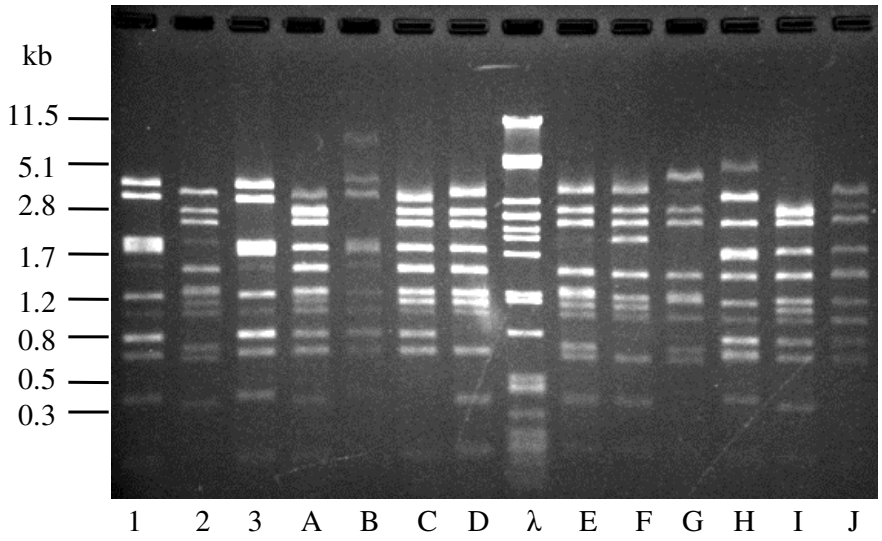

Figure 1.3. PCR-RFLP patterns of the *wla*I gene cluster digested with *Hha*I. λ, λ-DNA digested with *Pst*I; 1, WT O:4; 2, WT 81116; 3, O:4 Δ*wlaVA*; and A to J, transformants.

Identification of the integration point downstream of the *Cj1152c* gene

To identify the integration point downstream of the *Cj1155c* gene, *Hind*III-PCR-RFLP was performed. The primers Cj1155c-F and DnaX-R were used to amplify a 5.2-kb DNA fragment ranging from the *Cj1155c* gene to the *dnaX* gene by PCR resulting in amplification products of approximately 5.2 kb for WT O:4, O:4 Δ*wlaVA*, WT 81116, and 81116 transformants (B to J). A PCR product could not be amplified from transformant A. The integration point for transformants B, D, E, F, and H was located in or downstream of the *dnaX* gene since their RFLP patterns were identical to the RFLP pattern of O:4 Δ*wlaVA*. For transformants C, G, I, and J, the integration point was located in the *Cj1155c* gene as their RFLP patterns were identical to the pattern of WT 81116. The most upstream and downstream integration points for each 81116 transformant and the approximate sizes of WT 81116 DNA that were deleted during genetic recombination are conclusively shown in Figure 1.4.

Genotyping of 81116 transformants

To determine whether natural transformation with chromosomal DNA resulted in new genotypes of *C. jejuni*, pulsed-field gel electrophoresis (PFGE) with *Sac*II as the restriction enzyme was performed. The results showed that the *Sac*II-PFGE patterns of the transformants A, B, C, D, E, F, H, I, and J were identical to the PFGE pattern of WT 81116 (Fig. 1.5, see representative PFGE pattern of transformant A). Interestingly, the PFGE pattern of the transformant G was different from that of WT 81116. A large 388-kb DNA fragment and a 135-kb fragment of the O:4 Δ*wlaVA* were inserted into the chromosomal DNA of WT 81116. Presumably, this insertion is at a location other than the LOS gene cluster since the change involving the LOS genes is too small to be detected by PFGE. This indicated that not only the LOS gene cluster but also other locations throughout the WT 81116 genome had undergone a genetic exchange. These results showed that natural transformation can result in genotypic diversity, as also shown by previous studies (1, 11, 109).

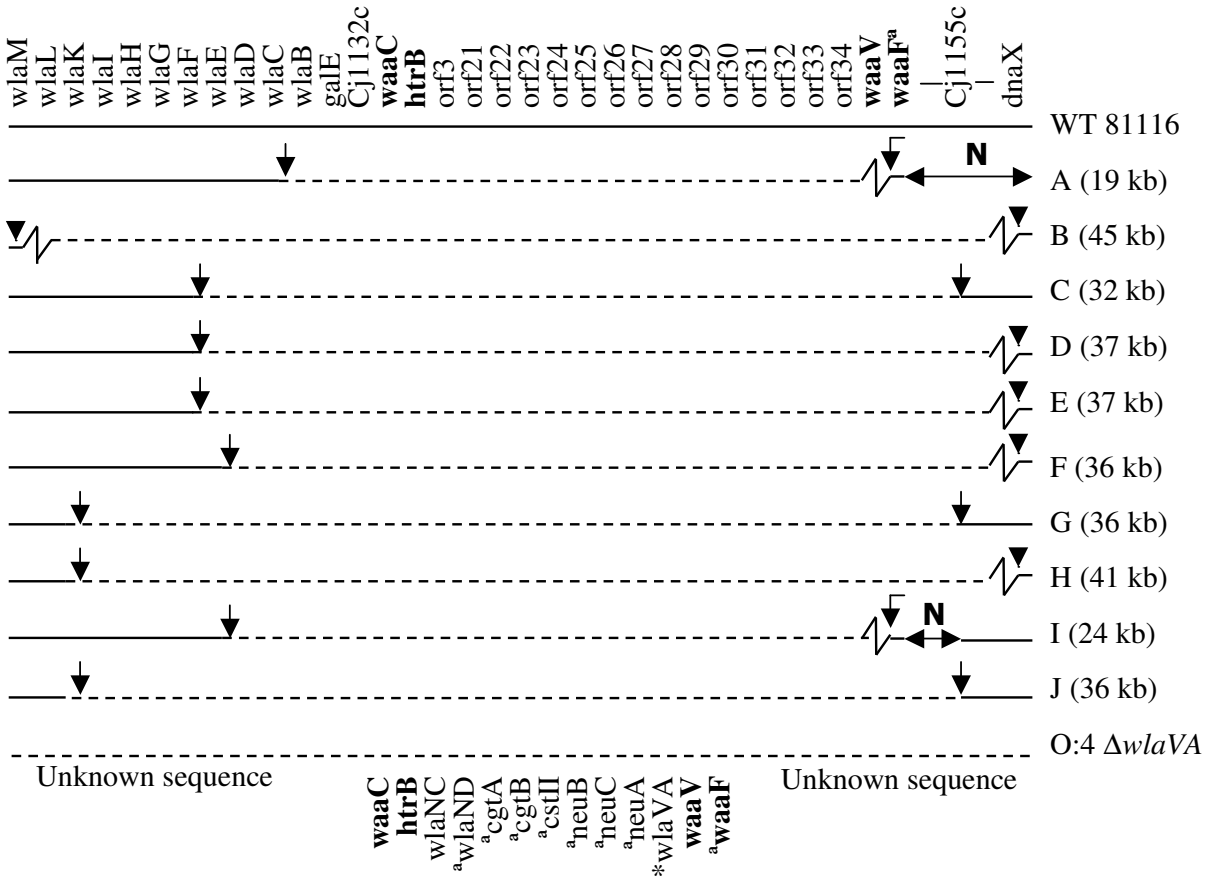

Figure 1.4. The *wla*I locus (*wlaM-Cj1132c*), the *wla*II locus (*waaC-waaF*) and its downstream region (*waaF-dnaX*), approximate DNA fragment sizes that were deleted from WT 81116 (numbers within parentheses), and integration points (arrows). WT 81116, host cell (GM1$^-$, accession no. Y11648, AF343914, and AJ131360, also see Figure 3 in the general introduction); O:4, donor cell (GM1$^+$, accession no. AF215659, also see Figure 3 in the general introduction); A to J, 81116 transformants exhibiting ganglioside GM1 (except transformant A); boldface letters, homologous gene regions; [a], essential genes for the formation of ganglioside-like LOS structures; dash between genes, unknown DNA regions; vertical arrows, the most upstream and downstream integration points; triangle point (left), integration point in or upstream of *wlaM*; elbow arrows and triangle points (right), integration points in or downstream of *waaF* and *dnaX*, respectively; N, not determined as no PCR product was obtained; *, integration point for kanamycin resistance cassette in O:4 Δ*wlaVA*.

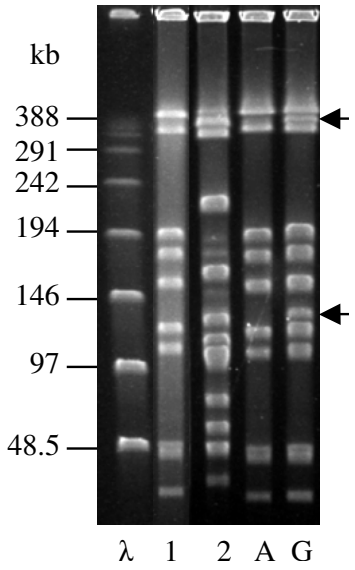

Figure 1.5. *Sac*II-PFGE patterns of the transformants. λ, molecular size marker (48.5-kb lambda concatemer); 1, WT 81116; 2, O:4 Δ*wlaVA*; A and G, transformants. Arrows indicate two DNA fragments of O:4 Δ*wlaVA* found in the transformant G.

LOS analysis of 81116 transformants

To examine the LOS molecules of transformants A to J, L, M, O, and Q, LOS was isolated and separated by Tricine-SDS-PAGE, followed by silver staining. The LOS patterns of all transformants were different from the LOS patterns of both WT 81116 and O:4 Δ*wlaVA*, except for LOS isolated from transformant Q that showed a similar LOS pattern to the WT 81116. Interestingly, the CTB-positive transformants (B, C, D, E, F, G, H, I, and J) carrying the LOS gene cluster of O:4 Δ*wlaVA* showed a different LOS pattern to that of O:4 Δ*wlaVA*. The cause for this difference is unclear and requires further investigation. As expected, the LOS patterns of WT O:4 and O:4 Δ*wlaVA* were similar (Fig. 1.6). These results indicated that not only the *wla*II gene cluster but also other genes seem to be involved in LOS synthesis.

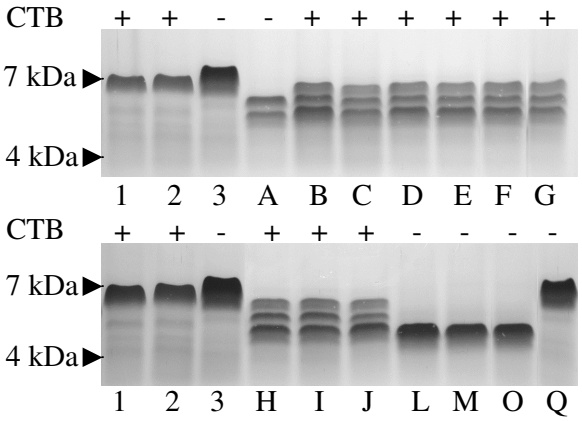

Figure 1.6. LOS analysis by Tricine-SDS-PAGE followed by silver staining. 1, WT O:4; 2, O:4 Δ*wlaVA*; 3, WT 81116; A to Q, transformants exhibiting CTB-positive (+) or -negative (−) phenotypes.

CONCLUSION

This study showed that a non-GM1 *C. jejuni* strain can take up DNA *in vitro* and transform into a number of potential GBS-inducing strains. Furthermore, we have shown that horizontal gene transfer between *C. jejuni* strains can result in genome plasticity. This result directly limits the current typing systems and complicates epidemiological studies. Moreover, since the incidence of mixed infections in areas of endemicity is high and could lead to the acquisition of virulence genes by non-pathogenic strains *via* interstrain genetic exchange, this result also indicates potential risks in using *C. jejuni* as a live vaccine in both animals and humans.

CHAPTER II

Evaluation of current RNA isolation methods for transcriptional analysis of the *wla*II-LOS synthesis gene cluster from *C. jejuni* strains

The study described in this chapter was accepted for publication in
Journal of Microbiological Methods, in press

ABSTRACT

DNA-free RNA samples are essential to investigate gene regulation using real-time RT-PCR. While attempting to analyse transcription of the lipooligosaccharide (LOS) synthesis gene cluster using RT-PCR, it was found that none of the published methods nor commercially available kits for RNA isolation could produce DNA-free RNA from *Campylobacter jejuni* strain HB 93-13. This study evaluated eight RNA isolation methods, including RNAzol B, Trizol, RNAgent, RNAwiz, RNeasy with DNase column, hot/SDS phenol RNA prep, and Triton X-100-based mRNA rich isolation and its modifications, for transcriptional analysis of the LOS-synthesis *neuC* gene from *C. jejuni* strain HB 93-13. RNA isolations were performed according to the published protocols and the manufacturers' instructions. Contaminating DNA was removed from RNA using a combination of TURBO DNase treatment and acid phenol extractions. Purified RNA samples were evaluated for purity and quantity using a spectrophotometer, quality by formaldehyde gel electrophoresis, and the presence of DNA by PCR and real time RT-PCR. The results showed that all methods yielded RNA with high purity levels. The highest RNA quantity was obtained with the Trizol and hot/SDS phenol RNA prep-based methods. The RNeasy with DNase column, Trizol, RNAwiz, and RNAzol B-based methods showed a high RNA quality. A DNA-free RNA sample could only be obtained using the RNAzolB-based method. The RNAgent, Trizol, and RNAwiz-based methods produced an RNA sample slightly contaminated with DNA, and the remaining methods showed high levels of DNA contamination. This study showed that a DNA-free RNA sample could be obtained for *C. jejuni* HB 93-13 by using RNAzolB in combination with TURBO DNase treatment and acid phenol extractions.

INTRODUCTION

Most researchers use either acid phenol extraction or DNase treatment to remove DNA from RNA samples. DNA decontamination is a crucial step for RNA preparation particularly when RNA samples are used for the investigation of gene regulation using real-time RT-PCR. When using acid phenol (pH 4-6) to remove DNA, DNA is retained in the organic phase and the interface, leaving the RNA in the aqueous phase (15). DNase is a non-specific endonuclease that degrades double-and single-stranded DNA. Presently, conventional DNase I and its variant, TURBO DNase, which has a greater catalytic efficiency than DNase I (Ambion), are widely used for DNA decontamination.

The efficiency of the removal of DNA from an RNA sample depends on the particular bacterial strain from which it is isolated. In a previous study DNA could be completely eliminated using DNase alone from an RNA sample isolated from *Escherichia coli* DH5α. In contrast, an RNA sample obtained from the Guillain-Barré syndrome-inducing *Campylobacter jejuni* strain HB 93-13 (40) still contained contaminating DNA after repeated treatment with DNase followed by acid phenol extraction. The aim of this study was to compare RNA isolation methods for their ability to produce DNA-free RNA, which could be used with real-time RT-PCR for transcriptional analysis of the lipooligosaccharide (LOS) synthesis gene cluster from *C. jejuni* strain HB 93-13.

MATERIALS AND METHODS

RNA isolation

C. jejuni strains 81116 (78), HB 93-13 (40), ATCC 43446, O:41, O:36, OH 4382 (3), 11168, PEN 4, and O:4 (a variant strain of PEN 4) were included in this study. Bacteria were grown on Columbia agar plates supplemented with 5% defribinated horse blood under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 42˚C for 16 h. The bacterial cells were harvested in cold PBS and washed once by centrifugation at 3,300 × g at 4˚C for 6 min. RNA was isolated using RNAzol B (Tel-Test), Trizol (Invitrogen), RNAgent (Promega), RNAwiz (Ambion), RNeasy with DNase column (Qiagen), Triton X-100-based mRNA-rich RNA isolation (99), and hot/SDS phenol RNA prep (http://www.bio.davidson.edu/projects/GCAT/protocols/ecoli/RNApurification.pdf) according to the published protocols or the manufacturers' instructions. In addition, a modified Triton X-100-based method was used in which acid phenol extraction (125:24:1, pH 4.5, Ambion) was used instead of a chloroform extraction.

RNA purification and analysis

RNA samples were treated with 10 U of TURBO DNase (Ambion) in a total volume of 700 µl at 37˚C for 2 h. After DNase treatment, the mixture was added with 700 µl of acid phenol (125:24:1, pH 4.5, Ambion), shaken vigorously for 20 s, incubated at room temperature for 15 min, and centrifuged at 16,100 × g at 4˚C for 15 min. The upper phase containing RNA was transferred into a new tube. The RNA was then precipitated by addition of 0.1 volume of 3 M sodium acetate (pH 5.2), 2.5 volumes of absolute ethanol, and incubation at -20˚C for 30 min. After centrifugation, the RNA pellet was washed once in 1 ml of 70% cold ethanol and centrifuged at 16,100 × g for 5 min. The RNA pellet was air-dried for 15 min and reconstituted with 100 µl of RNase-free water. The quantity of the purified RNA samples was determined by measuring the absorbance at 260 nm (A_{260}) using a spectrophotometer (Cary 50, Varian), the purity by measuring the A_{260}/A_{280} ratio, the quality by separating RNA

samples on a 2% formaldehyde gel as described in the RNeasy handbook, and the presence of contaminating DNA by PCR.

Reverse transcription

The cDNA synthesis was performed using the Omiscript reverse transcriptase kit (Qiagen). One microgram of RNA in a total volume of 13 μl was heated at 70°C for 3 min and immediately chilled on ice. The reaction mixture was prepared in a total volume of 20 μl, which consisted of 1 × RT buffer, 1 μg of heated RNA, 10 U of rRNasin (Promega), 50 ng of primer NeuC-R (5'-CCTTGAGCCAACTAAAATTC-3', nucleotide 8865-8884c, accession no. AY297047), 10 U of reverse transcriptase, and 500 μM of each dATP, dTTP, dCTP, dGTP. Reverse transcriptase was substituted by RNase-free water for the negative control. The RNA sample was substituted by genomic DNA isolated from *C. jejuni* HB 93-13 and used as the positive control. The reaction mixture was incubated at 37°C for 1 h and heated at 94˚C for 2 min. Ten μl of RNase A solution (50 μg/ml) was then added and the sample was incubated at 37˚C for 20 min.

Polymerase chain reaction

Two types of PCR were performed. The first of these was a general PCR and was carried out on a GeneAmp PCR system 2400 (Perkin Elmer) using *Taq* polymerase (ABI). The reaction mixture was prepared in a total volume of 50 μl, which contained 1 × *Taq* buffer, 1.5 mM of $MgCl_2$, 200 μM of each dATP, dTTP, dCTP, dGTP, 50 ng of each primer (NeuC-R as previously described and NeuC-F, 5'-GCCACGCTATATCAAAACTA-3', nucleotide 8379-8398, accession no. AY297047), 4 μl of reverse transcription product, and 2.5 U of *Taq* polymerase. PCR conditions were as follows: 94˚C for 3 min; 35 cycles of 94˚C for 30 s, 55˚C for 1 min, 72˚C for 1 min; and 72˚C for 7 min. The second type PCR was a quantitative PCR, which was performed on the MyiQ (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. PCR conditions were as follows: cycle 1 (1

×): 95˚C for 5 min; cycle 2 (35 ×): 95˚C for 30 s, 55˚C for 30 s, 72˚C for 30 s; cycle 3 (100 ×): 95˚C for 10 s (decrease setpoint temperature after cycle 2 by 0.5˚C, and enable melt curve data collection and analysis).

RESULTS AND DISCUSSION

Comparison of purity, quantity, and quality of RNA samples

To enable the purification of good quality DNA-free RNA from *C. jejuni* to study transcription, the RNeasy with DNase column, RNAgent, RNAwiz, RNAzolB, Trizol, Hot/SDS phenol RNA prep, and Triton X-100-based mRNA-rich RNA isolation and its modification in combination with TURBO DNase treatment and acid phenol extraction methods were compared for RNA purity, quantity, and quality. The results showed that all methods showed a high RNA purity as their A_{260}/A_{280} ratios were close to 1.8 (Table 2.1). Both the Trizol-based method and the hot/SDS phenol RNA prep-based method showed the highest RNA quantity (1.1 µg/µl), while the RNA yields for the other methods were close to 0.7 µg/µl (Table 2.1). A high RNA quality was obtained from the RNeasy with DNase column, RNAwiz, RNAzol B, and Trizol-based methods as these samples showed clear patterns on a formaldehyde gel. Interestingly, two patterns of total RNA were observed for different *C. jejuni* strains. The RNA pattern of strains 81116, PEN 4, and O:4 exhibited a normal pattern showing three separate bands, corresponding to 23S rRNA, 16S rRNA, and 5S rRNA as found in other Gram-negative bacteria (see RNeasy handbook). In contrast, the RNA pattern of strains HB 93-13, OH 4382, ATCC 43446, NCTC 11168, O:36, and O:41 revealed four bands (Fig. 2.1, selected result). It seems likely that the 23S rRNA fragment is cleaved resulting in two molecules, one larger and one smaller than 16S rRNA.

Table 2.1. Comparison of quantity and purity of RNA samples isolated by different methods

RNA isolation method	Total RNA (μg/μl)	A_{260}/A_{280}
Qiagen with DNase column	0.76	1.78
Hot/SDS phenol RNA prep	1.09	1.77
Triton X-100-based method	0.56	1.79
Modified Triton X-100 method	0.63	1.76
RNAgent	0.69	1.76
RNAwiz	0.74	1.79
RNAzol	0.71	1.76
Trizol	1.06	1.98

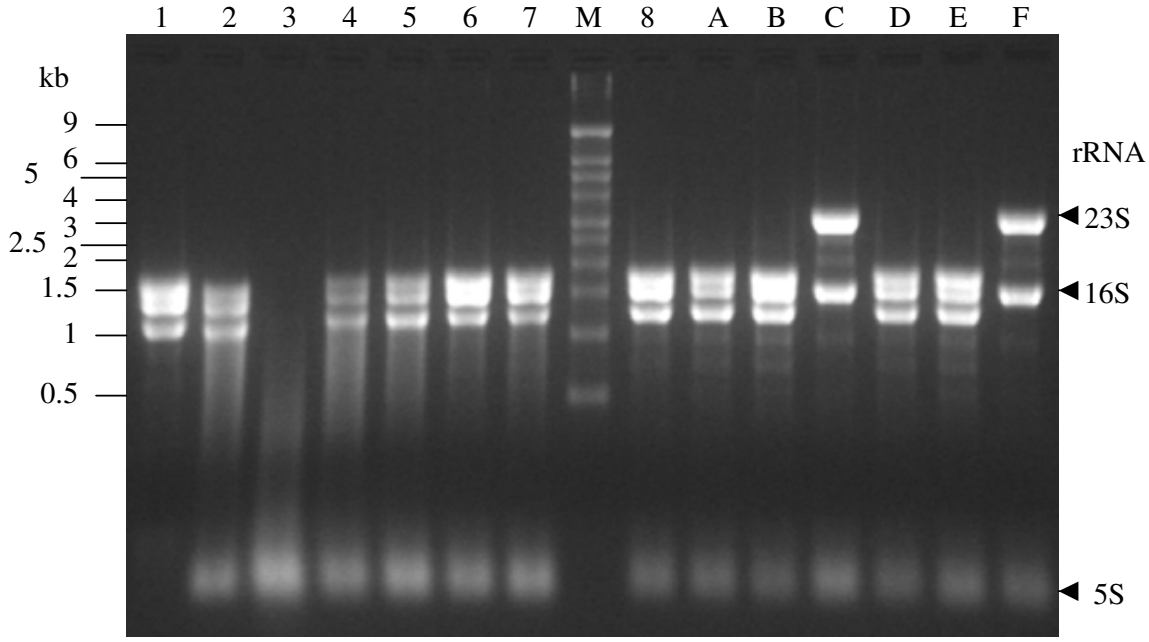

Figure 2.1. Comparison of RNA quality after separation on a 2% formaldehyde gel. M, RNA molecular weight marker (0.5-9 kb). 1-8, RNA samples isolated from *C. jejuni* HB 93-13 using different methods (1-8) in combination with TURBO DNase treatment and acid phenol extraction. 1, RNeasy with DNase column; 2, hot/SDS phenol RNA prep; 3, Triton X-100-based mRNA-rich RNA isolation; 4, modified triton X-100-based mRNA-rich RNA isolation; 5, RNAgent; 6, RNAwiz; 7, RNAzol B; and 8, Trizol. A-F show two forms of total RNA patterns among *C. jejuni* strains. A, ATCC 43446; B, NCTC 11168; C, NCTC 81116; D, O:36; E, O:41; and F, PEN 4. RNA samples (A-F) were isolated using the RNAzol B-based method. Arrows indicate the 5S, 16S, and 23S rRNA subunits.

Detection of contaminating DNA in RNA samples

Transcription of the *neuC* gene of *C. jejuni* HB 93-13 was analysed under normal growth conditions using RT-PCR and/or real-time RT-PCR. For RT-PCR, as expected, the *neuC*-specific cDNA fragments of approximately 500 bp were detected from all RNA samples isolated using the different RNA isolation methods. For the RNAzol B-based method, no RT-PCR product was obtained for the negative control without reverse transcriptase, showing the lack of DNA contamination in the RNA sample. However, for all other methods, the *neuC*-specific DNA fragments were detected in all negative controls, showing DNA contamination in these RNA samples (Fig. 2.2A). Similarly, the results of real time RT-PCR showed that only the RNAzol B-based method could produce DNA-free RNA, while the other methods showed high DNA contamination in RNA samples (result not shown). It was noted that after the amount of PCR template (reverse transcription product) was reduced from 4 µl to 1 µl, no PCR product was observed from the negative controls of the RNAgent, RNAwiz, and Trizol-based methods, showing that these methods produced RNA samples that were slightly contaminated with genomic DNA (result not shown). This is in contrast to the Qiagen with DNase column, Hot/SDS phenol RNA prep, Triton X-100-based mRNA-rich RNA isolation and its modification methods, which still showed high DNA contamination in the RNA samples (Fig. 2.2B). These results showed that DNA-free RNA samples could only be achieved using the RNAzol B-based method.

Strain specificity of DNA decontamination

To examine whether the severity of DNA contamination is strain-specific, *C. jejuni* strains HB 93-13, NCTC 11168, ATCC 43446, OH 4382, O:36, O:4, PEN 4, and O:41 were used to isolate RNA. The RNAzol B kit was used to prepare RNA samples of which some were exposed to both acid phenol extraction and TURBO DNase treatment to remove any remaining DNA. After DNA decontamination, all samples were treated with the RNase A enzyme as previously described. PCR was performed to amplify a fragment of the *neuC* gene

from these samples. The DNA sample isolated from *C. jejuni* strain 81116 without the *neuC* gene in its LOS synthesis gene cluster (accession no. Y11648 and AF343914) was used as the internal negative control. A DNA sample isolated from *C. jejuni* strain HB 93-13 was used as the positive control. No PCR product was observed using the RNase and DNase-treated RNA samples, while PCR products of 506 bp were detected from the RNase-treated RNA samples. As expected, a 500 bp PCR product was detected for the positive control, and no PCR product was observed for the internal negative control (Fig. 2.2C). These results showed that DNA decontamination was essential for all *C. jejuni* strains tested as all RNA samples contained DNA.

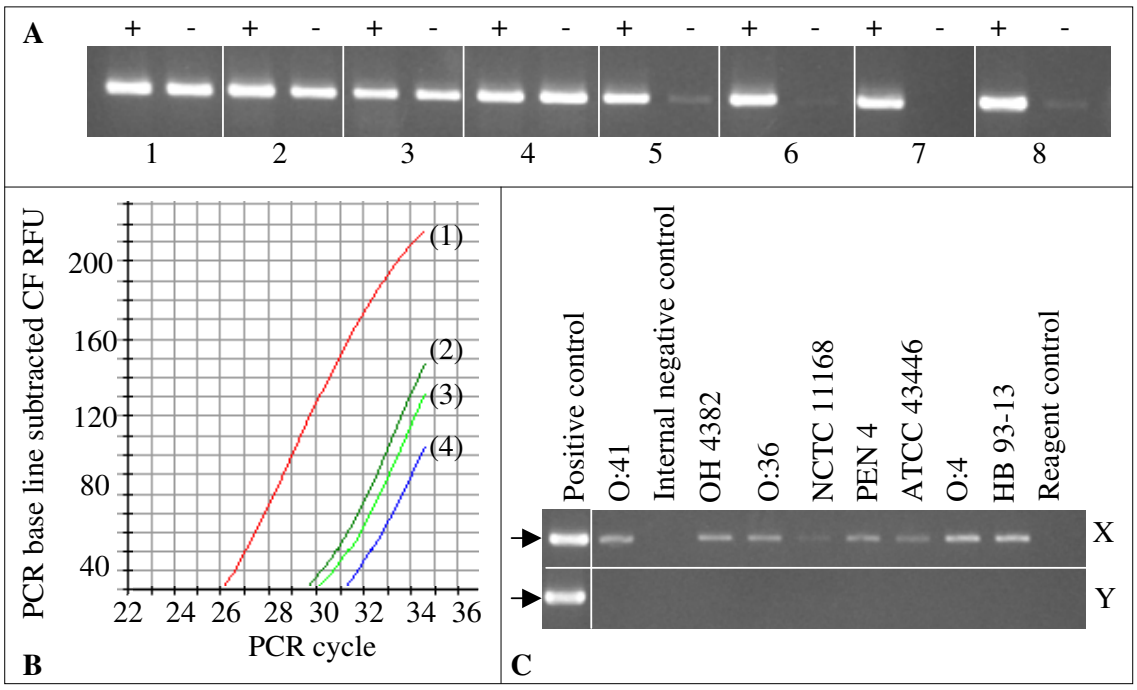

Figure 2.2. RT-PCR results. **A**, Detection of DNA contamination in RNA samples isolated by different methods (1-8) in combination with DNA decontamination methods (TURBO DNase treatment and acid phenol extraction). 1, RNeasy with DNase column; 2, hot/SDS phenol RNA prep; 3, Triton X-100-based mRNA-rich RNA isolation; 4, modified Triton X-100-based mRNA-rich RNA isolation; 5, RNAgent; 6, RNAwiz; 7, RNAzol B; and 8, Trizol. Plus (+), RT-PCR products of *neuC*-specific cDNA fragment (506 bp). Minus (-), RT-PCR products from the negative controls without reverse transcriptase, showing DNA contamination in RNA samples. **B**, comparison of the amount of contaminating DNA in the negative controls (1-4, as described in A) using quantitative real-time RT-PCR. **C**, Detection of DNA in RNA samples isolated from various *C. jejuni* strains using PCR. Arrows: *neuC*-specific PCR products (506 bp) in different *C. jejuni* strains. X, RNase-treated RNA sample; Y, RNase and DNase-treated RNA sample.

CONCLUSION

This study showed that most RNA isolation methods could not isolate RNA without DNA from *C. jejuni* strains. DNA-free RNA samples could only be isolated from *C. jejuni* using the combination of RNAzol B, TURBO DNase treatment, and acid phenol extraction. This difficulty might be caused by species-specific DNA conformation or chromatin structure. For example, very tight DNA packaging may render it insensitive to denaturation and separation. Moreover, two types of rRNA subunits were observed for different *C. jejuni* strains. Future investigations might reveal if these different types result in changes in protein synthesis or have some evolutionary advantage.

CHAPTER III

Transcription analysis of the *wla*II-LOS synthesis gene cluster from the GBS-inducing *C. jejuni* strain HB 93-13

The study described in this chapter was submitted for publication

ABSTRACT

The lipooligosaccharide (LOS) molecules of *Campylobacter jejuni* are involved in virulence and induction of the Guillain-Barré syndrome (GBS). This study analysed transcription of the LOS synthesis genes from the GBS-inducing *C. jejuni* strain HB 93-13 under microaerobic conditions. Fourteen consecutive genes *Cj1132c*, *waaC*, *htrB*, *wlaNC*, *wlaND*, *cgtA*, *cgtB*, *cstII*, *neuB*, *neuC*, *neuA*, *wlaVA*, *wlaQA*, and *waaF* were included. The results of rapid amplification of cDNA ends and single-stranded ligation of complementary ends showed initiation sites with potential promoter regions on both DNA strands in the *Cj1132c*/*waaC*, *cgtB*/*cstII*, and *wlaQA*/*waaF* strand-switch regions. Other termini without recognisable promoter region were also found throughout the LOS gene cluster, suggesting a low specificity of the polymerase during transcription. In addition, all gene junction regions were cloned into the shuttle vector pMW10 carrying the promoterless *lacZ* gene to identify functional promoter sites. Bidirectional active promoters were found in the strand-switch regions. The results of RT-PCR and cDNA blotting indicated that transcriptional linkage occurred between different operons, indicating a lack of transcription termination within the LOS gene cluster. Moreover, the results of semi-quantitative RT-PCR and real-time RT-PCR showed that both DNA strands were transcribed but transcription of the coding strand was at a higher rate, and both sense and antisense transcripts of each LOS gene examined were responsive to acid stress. The results presented here give a better insight into transcription of the LOS synthesis gene cluster of *C. jejuni*.

INTRODUCTION

The lipooligosaccharide (LOS) of *C. jejuni* is a major surface molecule consisting of two parts: the core oligosaccharide and the lipid A. It is involved in virulence (24) and induction of the Guillain-Barré syndrome (GBS), an autoimmune disorder of the peripheral nervous system (117). This crucial molecule is partly encoded by the *wla*II gene cluster that has been shown to exhibit a high degree of variation among strains (32, 80). Previously, Viraj and colleagues (74) cloned and sequenced the *wla*II-LOS synthesis gene cluster from the *C. jejuni* strain HB 93-13, which was originally isolated from a GBS patient in China (40). It contains 13 consecutive genes: *waaC*, *htrB*, *wlaNC*, *wlaND*, *cgtA*, *cgtB*, *cstII*, *neuC*, *neuB*, *neuA*, *wlaVA*, *wlaQA*, and *waaF* genes. This gene cluster is classified as a class A LOS gene cluster as previously described (32, 80). The *waaC* gene contributes to the synthesis of both LOS inner core and capsule carbohydrate (45). The *htrB* gene encodes a putative acyltransferase involved in lipid A synthesis. The *wlaND*, *cgtA*, *cgtB*, *cstII*, *neuB*, *neuC*, *neuA*, and *waaF* genes are essential for the formation of human ganglioside-like LOS structures, which can induce Guillain-Barré syndrome (35, 37, 62, 63, 76, 117). Mutating the *wlaVA* gene in a strain HB 93-13 did not affect the LOS structure, the LOS sugar composition, and the ganglioside mimicry (Viraj N. Perera, unpublished data). The *wlaNC* and the *wlaQA* gene are proposed to encode for a putative glucosyltransferase and a putative glycosyltransferase, respectively (32, 81). These studies, relating to the LOS gene cluster of *C. jejuni,* focussed on functional characterisation of the LOS genes, while little information on gene regulation is known. In this study the transcription of the LOS synthesis genes was analysed for *C. jejuni* strain HB 93-13.

MATERIALS AND METHODS

Bacterial strains and growth conditions

C. jejuni strains, including HB 93-13 (40), ATCC 43446, OH 4382 (3), NCTC 11168, 81116 (78), and all Penner serotype (1 to 60) strains and variant strain of Penner serotype 4 (Biotechnology laboratory, RMIT University, Australia), were grown on Columbia agar plates supplemented with 5% (v/v) defibrinated horse blood under microaerobic conditions at 37ºC for 16 h unless otherwise stated. *Escherichia coli* was grown in Luria-Bertani (LB) broth or agar at 37ºC for 16 h. Media were supplemented with kanamycin (50 μg/ml) when appropriate.

Primer design and definition

DNA sequence analysis was performed using Clone manager version 6 (Scientific and Educational Software, USA). Primers were designed using the LOS synthesis gene cluster of *C. jejuni* HB 93-13 (accession no. AY297047) unless otherwise indicated. All primers were purchased from Sigma Genosys. The primers, which were used to amplify gene-specific regions (approximate 500 bp), were named after the gene they were amplifying, such as WaaC-F and WaaC-R. The primers, which were used to amplify the gene junction regions (300 to 600 bp), were designated as co-primer such as Co-waaC-F and Co-htrB-R. The primers, which were used for real-time quantitative RT-PCR (103 to 128 bp), were named as q-primer such as q-waaC-F and q-waaC-R. All oligonucleotide primers used in this study are listed in Table 3.1.

Table 3.1. Oligonucleotide primers used for gene regulation studies

Name	Sequence	Name	Sequence
Cj1132c-F	GCTTTATTCCACGCCCGTTA†	Co-cgtA-R	TCCTTTATGCCCCTCCCCTA
Cj1132c-R	GTGAGAGTGTGCCAGATGTT†	Co-cgtA-F	TTGAAAGAACCAGGAGATTG
WaaC-F	CCGTGGTTTTGCAATTTATC†	Co-cgtB-R	ATTGTAGCGTTCTCTATGTG
WaaC-R	CCCACATGTATGAGTATGTT†	Co-cgtB-F	TGCTACTTTCACACCTTCAT
HtrB-F	AAAGAACGCGATAAATTGTC	Co-cstII-R	AGTTCGGTCTCATATTCTTG
HtrB-R	ATAAACAGGGATGATCAAAG	Co-cstII-F	CTTCTAGTGAGGCTTATGGA
WlaNC-F	TGTTTTGCCAAGTAATGCTA	Co-neuB-R	GCCTTACTCATCTCATCTTC
WlaNC-R	TTCATCTTCTTGCCAAATGT	Co-neuB-F	AAAAAGCAGCTAAGCAAGAG
WlaND-F	AGATGAACACTCACTCAATA	Co-neuC-R	CTAAAGCCTTATCGGTTGAA
WlaND-R	CTACACTTAATGCTCGATTT	Co-neuC-F	TTCCATCGCTTAGATTTGAG
CgtA-F	TGCTTATTCATCCCTTTTAG	Co-neuA-R	GCACTTTATCGATTGTAGTA
CgtA-R	TATAAGCCACTCATCTTTTG	Co-neuA-F	CGATTTAGCAGGGATTTGTA
CgtB-F	TTTAAAACCAACTGCAACTC	Co-wlaVA-R	AAACATAGAAACCCCATCAG
CgtB-R	AAGATATGAAGGTGTGAAAG	Co-wlaVA-F	AGGACAAAGAGCTGTAGTTA
CstII-F	ACCAAGCTCATCTAGAAAAT	Co-wlaQA-R	AGAATTATGGCGAAGATGTG
CstII-R	TCACTAGAAGGTATGAGTAT	Co-wlaQA-F	ACGCTATTTCACAAGCATCA
NeuB-F	AACCGCTTAGAAGATATGGG	Co-waaF-R	GCGTAAAGATAGGGCTTGTT
NeuB-R	TGTGACTTGCTCTTCTTAG	q-htrB-F	TTATGCCTGATTGTATCTTG
NeuC-F	GCCACGCTATATCAAAACTA	q-htrB-R	TTGAGTGTATTGAGGAAAAC
NeuC-R	CCTTGAGCCAACTAAAATTC	q-waaC-F	AATTAGCGCCAAAATTCAATC
NeuA-F	TTGTTGTAAGCAGTGATAGT	q-waaC-R	TTAAAGCAAAAGCCAAATGTG
NeuA-R	AAATCCTCCAAACAGTCAAT	q-cgtB-F	GAATTATTATTAGGCACGTAATG
WlaVA-F	TTCTTTTAGCGGATCTTTTC	q-cgtB-R	GTTAAAATCAATATGGCTGAAG
WlaVA-R	TCTTCTAGTAGGTCAAGATA	q-cstII-F	CTATATTGCTTATGTCCTAAC
WlaQA-F	GCCATAATTCAAACGCTCAT	q-cstII-R	ACTAGAAGGTATGAGTATATC
WlaQA-R	CTTGCGATTTTATCTCAAG	q-wlaQA-F	ATCTCTTTAAACGCTATTTCAC
WaaF-F	AAGATGCCCAGTTTATCCTT†	q-wlaQA-R	GAAGAAAATTTAGGCACTTTTG
WaaF-R	AAGTTCTTGTTCGGCTTTTC†	q-waaF-F	TTTATGGCTCTTTGGTTTC
Co-cj1132c-F	AGGCAAAGGCAAAAACTCAC†	q-waaF-R	TTCTTTGCGTAAAGATAGG
Co-waaC-R	TTTTAAGGGCAGGGCATAGA†	q-rpoA-F	GCTTTAGATGCTTTCTTTAC†
Co-waaC-F	AGGAAATGATAGCGGTCCAA	q-rpoA-R	ATTTGTCCATCAGTTGTTAC†
Co-htrB-R	AAAGCCAGGGCATCTATAAG	23S rRNA-F	TGAGCAAGTTGAAGCTAGTG†
Co-htrB-F	GAGCAAGCCTTATCGCACAA	23S-rRNA-R	AATAGAACGCTCTCCTACCA†
Co-wlaNC-R	CAAGCACTTCATCAGCATCA	SLIC1	NH_2-TCGTTTTTGTCGAGTTTGAG
Co-wlaNC-F	GAACATTTAGAATTTGCACAAAG	SLIC2	AAGGGCAGGGCATAGAGTT
Co-wlaND-R	GATAACGCTATCTAAACATTCTC	Anti-SLIC	TTCATAGCGGTCCACCGACA
Co-wlaND-F	TCGGGGAAAACGCTAAGGAA		
AAP	GGCCACGCGTCGACTAGTACGGGIIGGIIGGGIIG (Invitrogen)		
Linker	PO_4-ACTAGTGTCGGTGGACCGCTATGAATTCCTGTTGTGCCAAGCGATGATAC-NH_2		

†, Primers were designed using the genome of *C. jejuni* NCTC 11168 (accession no. AL111168), the other primers were designed using the LOS gene cluster sequence of *C. jejuni* HB 93-13 (accession no. AY297047).

Sequencing of the *Cj1132c* and *waaC* gene junction region

Using the genome sequence of *C. jejuni* NCTC 11168 (81), the primers Cj1132c-F and Co-htrB-R were designed to amplify a 1,911 bp DNA fragment containing the partial *Cj1132c*, entire *waaC*, and partial *htrB* genes. PCR was carried out by using *pfu* polymerase (Roche). The PCR conditions were as follows: 94ºC for 5 min; 35 cycles of 94ºC for 30 s, 55ºC for 1 min, 72ºC for 4 min; and 72ºC for 7 min. The PCR product was sequenced by using the ABI sequencing mix V 3.1 (ABI) according to the manufacturers' instructions.

Rapid amplification of cDNA ends (RACE)

The 5' RACE system version 2.0 (Invitrogen) was used to locate transcriptional start sites and promoters according to the manufacturer's instructions. Reverse transcription was carried out by using Omiscript reverse transcriptase (Qiagen) in which 5 μg of total RNA was used as the template for cDNA synthesis. All RACE primers used are described in Table 3.2. PCR was carried out by using *Taq* polymerase (ABI) as previously described. The PCR conditions were as follows: 95ºC for 6 min; 45 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s; and 72ºC for 7 min. A PCR product was cloned into the pCR2.1 vector using the TA cloning kit according to the manufacturer's instructions (Invitrogen). Plasmid DNA was isolated from white colonies using the mini-prep procedure as previously described (6) and digested by restriction enzymes to confirm positive clones. The pCR2.1 vector carrying the expected insert was purified using the QIAprep spin miniprep kit (Qiagen) and subsequently sequenced by using the ABI sequencing mix V 3.1 (ABI) according to the manufacturers' instructions.

Table 3.2. Primers used for mapping transcriptional start sites and promoter regions using RACE

Gene	RT and PCR primers†	RT and PCR primers§
Cj1132c	Not determined	Cj1132c-F and AAP/Co-cj1132c-F
waaC	WaaC-R and AAP/Co-waaC-R	WaaC-F and AAP/Co-waaC-F
htrB	HtrB-R and AAP/Co-htrB-R	HtrB-F and AAP/Co-htrB-F
wlaNC	WlaNC-R and AAP/Co-wlaNC-R	WlaNC-F and AAP/Co-wlaNC-F
wlaND	WlaND-R and AAP/Co-wlaND-R	WlaND-F and AAP/Co-wlaND-F
cgtA	CgtA-R and AAP/Co-cgtA-R	CgtA-F and AAP/Co-cgtA-F
cgtB	CgtB-R and AAP/Co-cgtB-R	CgtB-F and AAP/Co-cgtB-F
cstII	CstII-R and AAP/Co-cstII-R	CstII-F and AAP/Co-cstII-F
neuB	NeuB-R and AAP/Co-neuB-R	NeuB-F and AAP/Co-neuB-F
neuC	NeuC-R and AAP/Co-neuC-R	NeuC-F and AAP/Co-neuC-F
neuA	NeuA-R and AAP/Co-neuA-R	NeuA-F and AAP/Co-neuA-F
wlaVA	WlaVA-R and AAP/Co-wlaVA-R	WlaVA-F and AAP/Co-wlaVA-F
wlaQA	WlaQA-R and AAP/Co-wlaQA-R	WlaQA-F and AAP/Co-wlaQA-F
waaF	WaaF-R and AAP/Co-waaF-R	Not determined

†, RT and PCR primers used for mapping start site of sense transcript of each gene; §, RT and PCR primers used for mapping start site of antisense transcript of each gene.

Single-stranded ligation of complementary ends (SLIC)

The SLIC procedure used in this study was modified from a previous study (20). The first strand cDNA synthesis was carried out using the Omiscript reverse transcriptase (Qiagen), 3 µg of total RNA was used as the template. The primer SLIC1, which is located 535 bp downstream of the *waaC* start codon and contains the amine C3 group at its 5' end, was used as the anti-sense primer for cDNA synthesis. Primers and unincorporated dNTPs were removed from the reverse transcription product using the Geneclean kit according to the manufacturer's instructions. The 10 × ligation buffer was prepared as follows: 500 mM of Tris-HCL (pH 8.0), 100 mM of $MgCL_2$, 100 µg/ml of BSA, 10 mM of ATP, and 10 mM of hexamine cobalt chloride. This buffer was sterilized by filtration through a 0.22 µm membrane, and stored at -20ºC. The 3' end cDNA was ligated with the oligonucleotide linker that contains the phosphate group (PO_4) at the 5' end and the amine C3 group (NH_2) at the 3' end. The ligation reaction was prepared in a total volume of 10 µl containing 1 × ligation buffer, 100 ng of linker, 24% (v/v) of PEG 6000, 1 µl of purified cDNA, and 20 U of T4 RNA ligase (New England BioLabs). The reaction mixture was incubated at 22ºC for 48 h. The ligation product was purified using the Geneclean kit as previously described. The purified ligation product was used as the template for the nested PCR using primers Anti-SLIC and SLIC2. The anti-sense primer Anti-SLIC is complementary to the oligonucleotide linker. The sense primer SLIC2 is identical to the 5' cDNA end and is located 175 bp downstream of the *waaC* start codon. PCR was carried out using *Taq* polymerase (ABI). The PCR conditions were as follows: 94ºC for 5 min; 45 cycles of 94ºC for 30 s, 60ºC for 1 min, 72ºC for 1 min; and 72ºC for 7 min. The PCR product was cloned into the pCR2-1 vector and sequenced as previously described in the RACE method. All SLIC primers and linkers designed in this study are shown in Table 1.

Introducing putative promoters into a reporter gene plasmid in *C. jejuni*

Each gene junction region between the *Cj1132c* gene and the *waaF* gene was amplified from isolated genomic DNA of *C. jejuni* strain HB 93-13 using *Taq* polymerase (ABI). All co-primers used are listed in Table 3.3. PCR products were cloned into the PCR2.1 vector using the TOPO TA cloning kit (Invitrogen). Digestion of isolated plasmids with appropriate restriction enzymes was performed to confirm positive clones and determine the insert orientation. PCR2.1 carrying each gene junction region was digested with *Bam*HI/*Xba*I, ligated into *Bam*HI/*Xba*I-digested pMW10 carrying the promoterless *lacZ* gene (115), and introduced into chemically competent *E. coli* strain S17.1 by heat shock (91). The pMW10 carrying each gene junction region was confirmed by restriction mapping.

Various *C. jejuni* strains were tested for their ability to take up and host the pMW10 plasmid from *E. coli*. These bacterial strains included HB 93-13 (GBS), ATCC 43446, OH 4382 (GBS), NCTC 11168, NCTC 81116, variant strain of Penner serotype 4, and all Penner serotype (1 to 60) strains. Recombinant pMW10 plasmids were isolated from *E. coli* and introduced into various *C. jejuni* strains using electro-transformation (108) and/or natural transformation. For natural transformation, at least 10 µg of plasmid was mixed with 300 µl of *C. jejuni* cells suspended in Mueller-Hinton broth and the mixture was transferred onto Mueller-Hinton agar plates and incubated at 37˚C under microaerobic conditions overnight. After incubation, bacterial cells were harvested in Mueller-Hinton broth and grown on Columbia agar plates supplemented with 5% (v/v) defibrinated horse blood and kanamycin under microaerobic conditions at 42ºC for 48 h. The *C. jejuni* transformants carrying recombinant plasmids were confirmed by restriction mapping.

Table 3.3. Primers used for co-transcriptional analysis of LOS genes

Gene junction region	RT primers†	RT primers‡	PCR primers
Cj1132c/*waaC*	WaaC-R	Cj1132c-F	Co-cj1132c-F/Co-waaC-R
waaC/*htrB*	HtrB-R	WaaC-F	Co-waaC-F/Co-htrB-R
htrB/*wlaNC*	WlaNC-R	HtrB-F	Co-htrB-F/Co-wlaNC-R
wlaNC/*wlaND*	WlaND-R	WlaNC-F	Co-wlaNC-F/Co-wlaND-R
wlaND/*cgtA*	CgtA-R	WlaND-F	Co-wlaND-F/Co-cgtA-R
cgtA/*cgtB*	CgtB-R	CgtA-F	Co-cgtA-F/Co-cgtB-R
cgtB/*cstII*	CstII-R	CgtB-F	Co-cgtB-F/Co-cstII-R
cstII/*neuB*	NeuB-R	CstII-F	Co-cstII-F/Co-neuB-R
neuB/*neuC*	NeuC-R	NeuB-F	Co-neuB-F/Co-neuC-R
neuC/*neuA*	NeuA-R	NeuC-F	Co-neuC-F/Co-neuA-R
neuA/*wlaVA*	WlaVA-R	NeuA-F	Co-neuA-F/Co-wlaVA-R
wlaVA/*wlaQA*	WlaQA-R	WlaVA-F	Co-wlaVA-F/Co-wlaQA-R
wlaQA/*waaF*	WaaF-R	WlaQA-F	Co-wlaQA-F/Co-waaF-R

†, Primers used for cDNA synthesis from sense transcript containing each gene junction region; ‡, primers used for cDNA synthesis from antisense transcript containing each gene junction region.

To screen for the promoter-positive *C. jejuni* transformants, *C. jejuni* strains carrying a constructed reporter plasmid were grown at 42ºC under microaerobic conditions for 16 h. The gene junction region showing an active promoter in *C. jejuni* was demonstrated by testing the β-galactosidase activity. One full loop of bacterial cells was added to an Eppendorf tube containing 250 µl of 0.1% (w/v) X-Gal in PBS and incubated at 37ºC for at least 4 h. Active promoters gave the growth media a blue colour.

Assay of promoter activity

The *C. jejuni* strains showing functional promoters in the inserted gene junction regions were grown as previously mentioned. The bacterial cells were washed once with 1 ml of cold PBS without magnesium and calcium at 3,300 × *g* for 5 min. The pellet was resuspended in 500 µl of cold PBS, and cell density was determined by measuring the OD_{600}. After centrifugation as above, the pellet was suspended in 500 µl of lysis buffer [0.1M Tris-HCL (pH 7.8), 0.5% (v/v) Triton X-100] and incubated at 37˚C for 20 min. Cell debris was removed by centrifuging at 16,000 × *g* at 4ºC for 10 min, and the supernatant (cell lysate) was used for testing β-galactosidase activity, which was measured by conversion of p-nitrophenol-β-D-galactopyranoside (PNPG) as previously described (91, 115). The β-galactosidase assay was carried out in duplicate.

Northern blot analysis

DNA probes were prepared. Chromosomal DNA was isolated from *C. jejuni* HB 93-13 using the cetytrimethylammonium procedure (6), and 100 ng of DNA was used as the PCR template. PCR was carried out by using *pfu* polymerase (Roche) to amplify the gene junction regions using co-primers (Table 3.3). The PCR conditions were as follows: 94ºC for 5 min; 35 cycles of 94ºC for 30 s, 60ºC for 1 min, 72ºC for 1 min; and 72ºC for 7 min. The PCR products were gel-purified using the QIAquick gel extraction kit (Qiagen), and a 500 ng purified PCR product was labelled with digoxigenin-11-dUTP using the DIG labelling kit (Roche) according to the manufacturer's instructions.

Northern blot analysis was performed as described in the DIG system user's guide book (Roche) unless otherwise stated. Both total RNA and mRNA-rich samples were separated on a 1.5% (v/v) formaldehyde gel as described in the RNeasy handbook (Qiagen) and subsequently blotted onto a Hybond N membrane (Amersham) by upward capillary transfer in 20 × SSC buffer as previously described (91). The RNA fragments were fixed to the membrane by UV-crosslinking for 5 min. Pre-hybridisation buffer was prepared as follows: 7% (w/v) SDS, 50% (v/v) formamide, 5 × SSC, 0.1% (w/v) N-lauroyl sarcosine, 50 mM sodium dihydrogen orthophosphate monohydrate (pH 7.0), 2% (v/v) blocking reagent, and yeast tRNA (100 µg/ml). The mRNA-DNA hybridization was performed at 50ºC overnight. Detection was performed using the alkaline phosphatase-conjugated anti-DIG antibody and the NBT/BCIP substrate. The RNA ladder (0.5-9 kb, Ambion) was used as the standard marker. The 23S *rRNA* gene was used as the positive control.

RT-PCR

RNA isolation was performed using RNAzolB (Tel-Test), and the combination of TURBO DNase treatment (Ambion) and extraction with acid phenol (125:24:1, pH 4.5, Ambion) was used to remove contaminating DNA from the RNA sample according to the manufacturers' instructions. Reverse transcription was carried out by using Omiscript reverse transcriptase (Qiagen). All primers used for RT-PCR are described in Tables 3.3 and 3.4. One microgram of the purified total RNA sample was used as the template for cDNA synthesis. RNase-free water was substituted for reverse transcriptase for the negative control. Genomic DNA of *C. jejuni* HB 93-13 was used instead of RNA for the positive control. After cDNA synthesis, 10 µl of RNase A solution (20 µg/ml) was added to the samples and they were incubated at 37°C for 20 min. After the RNase treatment, two types of PCR were performed. The first one, a conventional PCR, was carried out in a GeneAmp PCR system 2400 (Perkin Elmer) using *Taq* polymerase (ABI) and/or *pfu* polymerase (Roche) according to the manufacturers' instructions. The PCR conditions were programmed on the GeneAmp 2400 (Perkin Elmer) as

Table 3.4. Gene-specific primers used for transcriptional analysis of individual genes

Genes	RT primers†	RT primers‡	PCR primers
Cj1132c	Cj1132c-R	Cj1132c-F	Cj1132c-F/Cj1132c-R
waaC	WaaC-R	WaaC-F	WaaC-F/WaaC-R
htrB	HtrB-R	HtrB-F	HtrB-F/HtrB-R
wlaNC	WlaNC-R	WlaNC-F	WlaNC-F/WlaNC-R
wlaND	WlaND-R	WlaND-F	WlaND-F/WlaND-R
cgtA	CgtA-R	CgtA-F	CgtA-F/CgtA-R
cgtB	CgtB-R	CgtB-F	CgtB-F/CgtB-R
cstII	CstII-R	CstII-F	CstII-F/CstII-R
neuB	NeuB-R	NeuB-F	NeuB-F/NeuB-R
neuC	NeuC-R	NeuC-F	NeuC-F/NeuC-R
neuA	NeuA-R	NeuA-F	NeuA-F/NeuA-R
wlaVA	WlaVA-R	WlaVA-F	WlaVA-F/WlaVA-R
wlaQA	WlaQA-R	WlaQA-F	WlaQA-F/WlaQA-R
waaF	WaaF-R	WaaF-F	WaaF-F/WaaF-R
23S rRNA	Not determined	Not determined	23S-rRNA-F/23S-rRNA-R

†, Primers used for cDNA synthesis from sense transcript of each gene; ‡, primers used for cDNA synthesis from antisense transcript of each gene.

follows: 94°C for 3 min; 35-40 cycles of 94°C for 30 s, 58-60°C for 1 min, 72°C for 1 min; and 72°C for 7 min. The second type, a semi-quantitative PCR, was performed as previously described, except that the PCR conditions were as follows: 94°C for 3 min; 25-35 cycles of 94°C for 30 s, 45°C for 1 min, 72°C for 1 min; and 72°C for 7 min.

Southern blot analysis

Southern blot analysis was performed as described in the DIG system user's guide book (Roche) unless otherwise stated. Briefly, RT-PCR products were separated on a 2.5 % (w/v) agarose gel and blotted onto a Hybond N membrane (Amersham) by upward capillary transfer in 20 × SSC buffer overnight as previously described (91). The cDNA fragments were fixed to the wet membrane by UV-crosslink for 5 minutes. Pre-hybridization buffer was prepared as follows: 5 × SSC, 0.1% (w/v) N-lauroyl sarcosine, 0.02% (w/v) SDS, and 1% (v/v) blocking reagent. DNA probes were those described in northern blot analysis. The cDNA-DNA hybridisation was performed at 68°C overnight. Detection of hybridisation was performed as described for northern blot analysis.

Treatment of *C. jejuni* with acid stress and RNA preparation

C. jejuni HB 93-13 was grown in 30 ml of brucella broth with gentle shaking under microaerobic conditions at 37ºC for 19 h, and 100 µl of bacterial culture was aliquoted into 2 bottles of brucella broth (30 ml) and incubated for another 19 h. After incubation, 1 ml of brucella broth, which had been supplemented with concentrated HCl and pre-warmed at 37ºC, was added to the first culture bottle to obtain a final pH of 5.5 (acid stress). A pH of 5.5 was selected as acid stress since *C. jejuni* showed normal growth in brucella broth at pH 6.0, inhibited growth at pH 5.5, and no growth at pH 5.0. The second culture bottle, which had been added with 1 ml of pre-warmed brucella broth, was used as the calibrator (normal gene expression level). The incubation was performed at 37ºC with gentle shaking under microaerobic conditions. Culture samples were collected after incubation for 15 min and

transferred directly into a 1/10 volume of cold 10 × stop solution [5% (v/v) phenol in 100% ethanol] to halt transcription and RNA degradation. RNA samples were isolated using the RNAgents total RNA isolation system (Promega), and DNA decontamination was performed using the TURBO DNA-*free* Kit (Ambion).

Quantitative real-time RT-PCR

Both forward and reverse primers were separately used for reverse transcription. Primers q-htrB-R, q-waaC-R, q-cgtB-R, q-cstII-R, q-wlaQA-R, q-waaF-R, or q-rpoA-R were used for reverse transcription for quantification of forward transcripts of the *htrB*, *waaC*, *cgtB*, *cstII*, *wlaQA*, *waaF*, or *rpoA* genes, respectively. Primers q-htrB-F, q-waaC-F, q-cgtB-F, q-cstII-F, q-wlaQA-F, q-waaF-F, or q-rpoA-F were used for reverse transcription for quantification of reverse transcripts of the *htrB*, *waaC*, *cgtB*, *cstII*, *wlaQA*, *waaF*, or *rpoA* genes, respectively.

The cDNA synthesis was performed using the SuperScript II reverse transcriptase (Invitrogen). One microgram of RNA, 50 ng of q-primer either sense primer or anti-sense primer, and 1 µl of dNTP mix (10 mM each) in a total volume of 12 µl was heated at 65°C for 5 min and immediately chilled on ice for at least 5 min. For each gene, dilution series of newly synthesized cDNA was performed and included in quantitative PCR to examine the efficiency of PCR. The master mix was prepared in a total volume of 7 µl, which consisted of 4 µl of 5 × first-strand buffer, 2 µl of 0.1 M DTT, and 1 µl of RNase-free water. The master mix was distributed into the reaction tube containing the mixture of heated RNA, primer, and dNTP. The tube was gently mixed and incubated at 42°C for 2 min followed by addition of 1 µl of revere transcriptase (200 u/µl). Reverse transcriptase was substituted by RNase-free water for the negative control. The reaction mixture was gently mixed, incubated at 42°C for 50 min, and heated at 70°C for 15 min. After cDNA synthesis, 10 µl of RNase A solution (20 µg/ml) was added and the samples were incubated at 37°C for 20 min and followed by addition of 220 µl of water.

Quantitative PCR was performed on the MyiQ (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The PCR reaction was performed in a total volume of 20 µl consisting of 10 µl of iQ SYBR Green Supermix and 10 µl of master mix, which consisted of 1 µl of forward primer (50 ng/µl), 1 µl of reverse primer (50 ng/µl), 3 µl of diluted cDNA solution, and 5 µl of water. Primers q-htrB-F/q-htrB-R, q-waaC-F/q-waaC-R, q-cgtB-F/q-cgtB-R, q-cstII-F/q-cstII-R, q-wlaQA-F/q-wlaQA-R, q-waaF-F/q-waaF-R, or q-rpoA-F/q-rpoA-R were used for amplifying the cDNA-specific fragments for the *htrB*, *waaC*, *cgtB*, *cstII*, *wlaQA*, *waaF*, or *rpoA* gene, respectively. Each PCR was performed in duplicate. The samples that showed a difference in cycle threshold ($C_T > 1$) were repeated. PCR conditions were as follows: cycle 1 (1 ×): 95°C for 5 min; cycle 2 (40 ×): 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; cycle 3 (100 ×): 95°C for 10 s (decrease setpoint temperature after cycle 2 by 0.5ºC, and enable melt curve data collection and analysis). Each specific amplicon was verified both by the presence of a single melting temperature peak and by the presence of a single band of expected size on a 3% agarose gel after electrophoresis. Cycle threshold values were determined by the MyiQ software (Bio-Rad). The relative changes (X-fold) in gene transcription between the induced and calibrator samples were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (64). Two internal control genes (*rpoA* and *htrB*) were included. The *rpoA* gene, which encodes the alpha subunit of RNA polymerase, was used as internal negative control as its expression was found to be constant under different environmental conditions (59, 97). The *htrB* gene, which encodes a putative acyltransferase involved in lipid A synthesis, was used as the internal positive control as its expression was highly up regulated under acid stress (Chapter IV). Relative expression values of $-2 < X > 2$ were considered to be significantly down or up regulated.

RESULTS

Identification of transcriptional start sites and promoter regions

To locate the start site and promoter region upstream of the *waaC* start codon on the upper DNA strand, 5' RACE was performed. The antisense primer WaaC-R, which is located 553 bp downstream of the *waaC* start codon, was used for cDNA synthesis. The homopolymeric dCTP tract was tailed to the 3' cDNA end by the terminal deoxynucleotidyl transferase enzyme. Nested PCR with primers AAP and Co-waaC-R was performed to amplify the dCTP-tailed cDNA. After amplification, the amplicon was cloned into the PCR2.1 vector and subsequently sequenced. For identification of transcriptional start sites and promoters for other LOS genes on both upper and bottom DNA strands, the same RACE principle was applied (Table 2). Sequence analysis of several cDNA clones identified multiple 5' termini for both the bottom and the top strands. Multiple start sites were observed for the genes *waaC* [26 bp upstream of the start codon at nucleotide A (-26, A), (-12, A), 37 bp downstream of the start codon at nucleotide A (+37, A), (+58, T), (+96, T)], *cgtB* [(-22, A), (+36, C), (+48, T)], *cstII* [(-27, A), (-17, C), (+19, G), (+110, A)], and *wlaVA* [(+74, C), (+96, A), (+111, A), (+199, T)]. One start site was observed for the genes *Cj1132c* (-25, C), *cgtA* (-23, A), *neuB* (+114, T), *wlaQA* (-27, T), and *waaF* (-31, A). Although multiple start sites were identified, even for a single gene, recognizable promoter regions were only located upstream of the most upstream initiation sites for the *Cj1132c*, *waaC*, *cgtA*, *cgtB*, *cstII*, *wlaQA*, and *waaF* genes (Table 3.5, also see Fig. 3.1A).

To confirm the start sites, which were previously inferred by the RACE method, SLIC was performed. A previous study showed that S1 nuclease protection, primer extension, and SLIC all identified multiple potential start sites of the human β1 subunit gene. However among these positions, the major transcriptional start site that was inferred from SLIC corresponded to the most upstream site (90). In this study, the start site upstream of the *waaC* start codon was selected to be confirmed. Multiple cDNA clones were observed, which indicated multiple

Table 3. 5. Promoter regions, initiation sites, ribosomal binding sites (RBS), and start codons (SC) in the LOS gene cluster of *C. jejuni* HB 93-13

Gene	Promoter region	+1	Gap	RBS	Gap	SC
waaC	tttgccatttt**a**acctttttttaaataatttcgcta**a**aatcatagc	a	16	aggct	4	atg
cgtA	taataaaaata**t**aaaaaattaattaatttttaggta**t**aatcactat	a	5	aggag	12	atg
cstII	ttattataattt**a**attataacataaaatctattttga**t**aaaatcgtt	a	14	tggaa	7	atg
waaF	gatagaaagt**t**gtggcatatttttcctaaattttg**t**taaaataat	a	21	aggaa	4	atg
Cj1132c*	ctattttcattaaaa**g**cctaatattttaaaatttttgcta**t**gattttagc	g	15	aggtt	4	atg
cgtB*	aagatttatttttaacg**a**ttttatcaaaatagattttatgtt**a**taattaaat	t	11	aggaa	5	atg
wlaQA*	aggtgtataaaaatt**t**tcataaatttcctaaactttgatag**a**attgttttt	a	17	aggaa	5	atg

*, Inverted and complementary strands shown; underlined letters indicate the putative -10 region; a bold letter (left) is the nucleotide at the -35 position; a bold letter (right) is the nucleotide at the -10 position; +1, is the most upstream transcriptional start site.

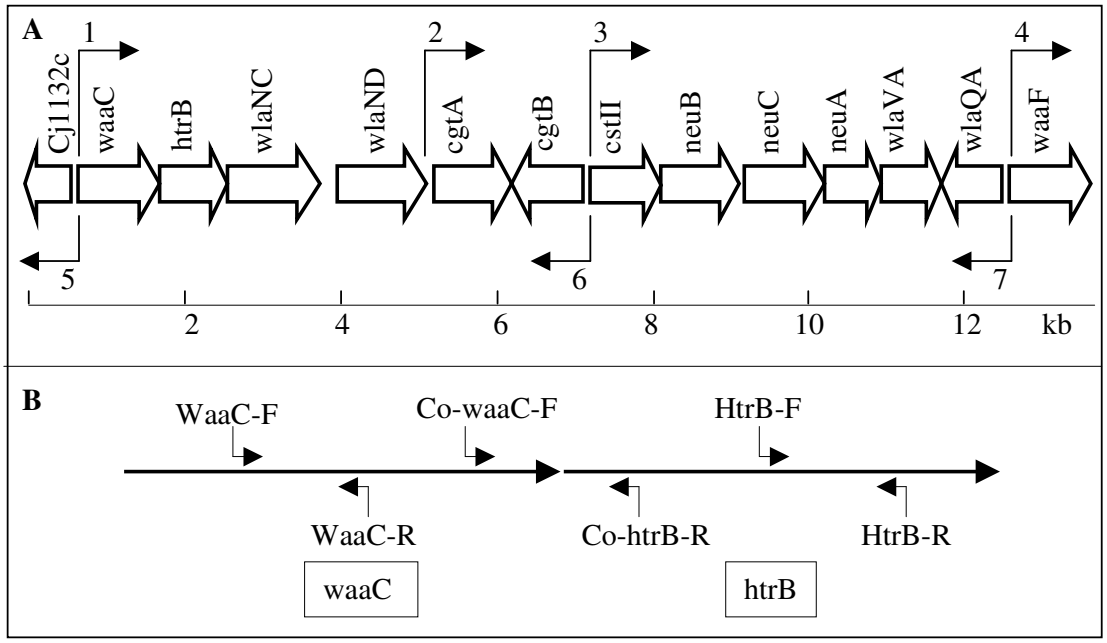

Figure 3.1. *wla*II-LOS synthesis gene cluster of *C. jejuni* HB 93-13 (**A**; accession no. AY297047) and location of primers (**B**). For **A**, open arrows indicate predicted transcription direction for each gene; elbow arrows with numbers indicate transcriptional start sites and their transcription directions. **B**, example of primer distribution along the *waaC* and *htrB* genes. Elbow arrows indicate primers used in this area.

start sites. In this experiment, only the clones carrying the largest cDNA fragments were sequenced as these most likely indicated the most upstream initiation site. The results showed that the most upstream start site was located at nucleotide A, 26 bp upstream of the *waaC* start codon (-26, A). This position is identical to the transcriptional start site upstream of the *waaC* start codon that was previously identified by the RACE method.

Functional characterisation of promoter regions

Individual gene junction regions were amplified from genomic DNA of *C. jejuni* HB 93-13 and then ligated into the pMW10 plasmid carrying the promoterless *lacZ* gene (115). The ligation product was initially introduced into various *C. jejuni* strains (see method). Transformation was performed using natural transformation and electro-transformation. Several attempts were made without success. Therefore an alternative approach was taken in which we cloned each gene junction region into the pMW10 plasmid and introduced the resulting constructs into *E. coli*. Following verification of the correct constructs they were purified from *E. coli* and introduced into various *C. jejuni* strains, again using both natural transformation and electro-transformation. However, again none of the *C. jejuni* strain tested was found to be able to host the pMW10 plasmid. Possibly, *C. jejuni* and *E. coli* harbour different restriction modification systems, which creates a barrier for interstrain plasmid transfer (2). Additional *C. jejuni* strains, all Penner serotype (1 to 60) strains, were tested for their ability to take up and host the recombinant pMW10 plasmids from *E. coli* using natural transformation. Only *C. jejuni* Penner serotype 59 could be transformed with the recombinant pMW10 plasmids. Therefore, this strain was used as the recipient for interstrain plasmid transfer. The β-galactosidase activity assay showed that the *Cj1132c*/*waaC*, *cgtB*/*cstII*, and *wlaQA*/*waaF* gene junction regions possessed functional promoters. Among these, the *wlaQA*/*waaF* region showed the strongest promoter strength (Table 3.6, 13.12A). This is consistent with our previous results that identified functional promoters located in the same regions where the transcriptional start sites and the potential promoters were inferred using

Table 3.6. β-Galactosidase activity of the *C. jejuni* transformants

Transfo -rmant	Junction region cloned in pMW10	Location*	Direction	Colony color	Miller unit
1.2D	*Cj1132c/waaC*	126450-126859†	S	Dark blue	320
2.3H	*waaC/htrB*	142-700	S	White	1
2.4A	*waaC/htrB*	142-700	O	White	1
3.1B	*htrB/wlaNC*	1009-1526	O	White	2
4.1A	*wlaNC/wlaND*	2321-2876	O	White	2
5.1W	*wlaND/cgtA*	3691-4255	O	White	5
6.1A	*cgtA/cgtB*	4733-5223	O	White	3
6.4H	*cgtA/cgtB*	4733-5223	S	White	4
7.1A	*cgtB/cstII*	5732-6268	S	Dark blue	419
8.1A	*cstII/neuB*	6799-7126	O	White	3
9.1D	*neuB/neuC*	7756-8204	O	White	1
9.2A	*neuB/neuC*	7756-8204	S	White	1
10.1B	*neuC/neuA*	8757-9377	O	White	2
10.13A	*neuC/neuA*	8757-9377	S	White	4
11.1A	*neuA/wlaVA*	9528-10052	O	White	3
12.1C	*wlaVA/wlaQA*	10277-10846	O	White	5
12.2A	*wlaVA/wlaQA*	10277-10846	S	White	4
13.1A	*wlaQA/waaF*	11072-11646	O	Light blue	41
13.12A	*wlaQA/waaF*	11072-11646	S	Dark blue	570
14	pMW10 without insert	1-10266‡	-	White	5

* , Location in the LOS gene cluster of *C. jejuni* HB 93-13 (accession no. AY297047); †, DNA sequence for the *Cj1132c/waaC* region was taken from the genome of *C. jejuni* NCTC 11186 (accession no. AL111168) and is identical to that of *C. jejuni* HB 93-13 (data not shown); ‡, entire nucleotide sequence of pMW10 (accession no. AJ001494); S, indicates the same orientation of the insert and the *lacZ* gene; O, indicates the opposite orientation of the insert relative to the *lacZ* gene.

RACE and/or SLIC methods. Plasmids showing no activity were sequenced to verify that no mutations were introduced during PCR and cloning. Plasmid sequence analysis showed that no mutations were present in the promoter-negative regions. Furthermore, the sizes of the inserts that were cloned into the pMW10 (Table 3.6) were large enough for the promoter activity assay to be successfully performed as previously shown (115). Therefore, it is likely that the other LOS gene junction regions, except *Cj1132c*/*waaC*, *cgtB*/*cstII*, and *wlaQA*/*waaF*, did not possess a functional promoter. The above conclusions were made assuming that the *C. jejuni* Penner serotype 59 and *C. jejuni* strain HB 93-13 polymerases recognise the same promoter region.

Transcriptional analysis of LOS genes

To estimate the transcript sizes, Northern blot analysis was performed. No hybridisation was observed for the tested LOS genes, while the positive control, the *23S rRNA* gene, did show hybridisation (result not shown). Several attempts were made without success. The lack of success was probably due to low amounts of LOS mRNA and/or rapid degradation of the LOS mRNA. Therefore, an alternative method for transcription analysis, RT-PCR, was used.

To determine co-transcription of the LOS genes nested RT-PCR was performed. As expected, no PCR product was observed for the negative controls without reverse transcriptase, confirming the lack of artifacts and DNA contamination in the RNA samples. For the forward direction the results showed that cDNA fragments containing the gene junction regions of *waaC*/*htrB*, *htrB*/*wlaNC*, *wlaNC*/*wlaND*, *wlaND*/*cgtA*, *cgtA*/*cgtB*, *cgtB*/*cstII*, *cstII*/*neuB*, *neuB*/*neuC*, *neuC*/*neuA*, *neuA*/*wlaVA*, *wlaVA*/*wlaQA*, and *wlaQA*/*waaF* were present (Fig. 3.2A). As expected, the cDNA fragment containing the *Cj1132c*/*waaC* gene junction region was not detected. This result indicated that the LOS genes (*waaF* to *waaC*) were co-transcribed in the forward direction. For the reverse direction the cDNA fragments containing the gene junction regions of *waaF*/*wlaQA*, *wlaQA*/*wlaVA*, *wlaVA*/*neuA*, *neuA*/*neuC*, *neuC*/*neuB*, *neuB*/*cstII*, *cstII*/*cgtB*, *cgtB*/*cgtA*, *cgtA*/*wlaND*, *wlaND*/*wlaNC*, *wlaNC*/*htrB*,

htrB/*waaC*, and *waaC*/*Cj1132c* were detected (Fig. 3.2B and 3.2C). This result indicated that the LOS gene cluster (*waaF* to *waaC*) and its adjacent *Cj1132c* gene were co-transcribed in the reverse direction.

To determine transcription of individual LOS genes, RT-PCR was performed. The results showed that the *Cj1132c* gene was transcribed in the reverse direction only as previously predicted (Fig. 3.1A). The other genes, including *waaC*, *htrB*, *wlaNC*, *wlaND*, *cgtA*, *cgtB*, *cstII*, *neuB*, *neuA*, *wlaVA*, *wlaQA*, and *waaF*, were transcribed in both forward and reverse directions since both sense and antisense transcripts for each gene were detected. Since the sizes of the RT-PCR products for sense and antisense transcripts for each gene were approximately 500 bp (see primer design), only the forward transcripts are shown (Fig. 3.2D).

To verify the sense and antisense transcripts indicated by RT-PCR, Southern blot analysis was performed. All RT-PCR products obtained from co-transcriptional analysis of LOS genes in the reverse direction were selected for confirmation. DNA probes containing the gene junction regions were used for hybridisation. The results showed specific hybridisation between DNA probes and RT-PCR products, confirming the lack of non-specific RT-PCR products (Fig. 3.2E).

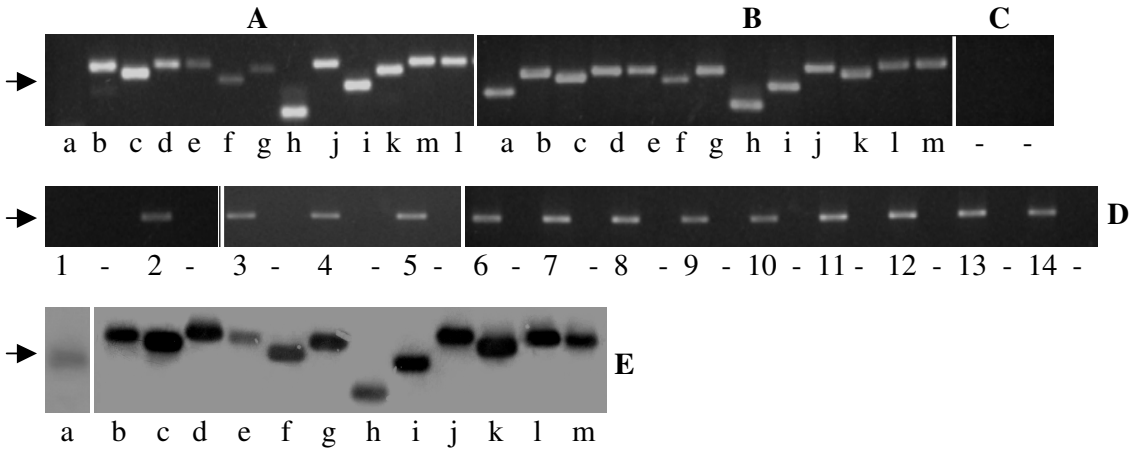

Figure 3.2. Transcriptional analysis of LOS genes. Arrows indicate 500 bp position. **A**, co-transcriptional analysis of genes in the forward direction by nested RT-PCR; a to m, transcripts containing gene junction regions of *Cj1132c*/*waaC*, *waaC*/*htrB*, *htrB*/*wlaNC*, *wlaNC*/*wlaND*, *wlaND*/*cgtA*, *cgtA*/*cgtB*, *cgtB*/*cstII*, *cstII*/*neuB*, *neuB*/*neuC*, *neuC*/*neuA*, *neuA*/*wlaVA*, *wlaVA*/*wlaQA*, and *wlaQA*/*waaF*, respectively. **B**, co-transcriptional analysis of genes in the reverse direction by nested RT-PCR; a to m, transcripts containing the same gene junction regions as described in A. **C**, representative negative controls without reverse transcriptase (-) for A and B. **D**, transcriptional analysis of individual genes in the forward direction by RT-PCR; 1 to 14, transcripts of the *Cj1132c*, *waaC*, *htrB*, *wlaNC*, *wlaND*, *cgtA*, *cgtB*, *cstII*, *neuB*, *neuC*, *neuA*, *wlaVA*, *wlaQA*, and *waaF* genes, respectively; minus (-) after each number indicates negative control for each gene. **E**, verification of RT-PCR products obtained from B (a to m) by Southern blot analysis.

Quantification of both sense and antisense transcripts of each LOS gene

To quantify the amount of both sense and antisense transcripts of each LOS gene, the combination of semi-quantitative RT-PCR and quantitative real-time RT-PCR was used. For semi-quantitative RT-PCR, all LOS genes were included. For quantitative real-time RT-PCR, the *waaC*, *cgtB*, *cstII*, *wlaQA*, and *waaF* genes were selected. This selection was based on the previous results that these genes possessed the functional promoters upstream of their start codons. Before the quantitative data were accepted, three criteria were required. Firstly, no PCR product should be detected from the negative control without reverse transcriptase, confirming the lack of DNA contamination in the RNA sample. Secondly, the PCR efficiency for each gene should be at least 80%. This ensured that low amounts of PCR product were due to low expression levels of the genes of interest and not due to low PCR efficiency. Thirdly, only one peak for each gene analyzed should be observed in the melting curve, confirming the lack of non-specific PCR product. The results showed that the major transcripts of the *waaC*, *htrB*, *wlaNC*, *wlaND*, *cgtA*, *cstII*, *neuB*, *neuA*, *wlaVA*, and *waaF* genes were found in the forward direction as more PCR product was obtained for their sense transcripts than for their antisense transcripts while the major transcripts of the *Cj1132c*, *cgtB*, and *wlaQA* genes were found in the reverse direction (Fig. 3.3 and Table 3.7). Similarly, the major transcription directions for all of these genes were consistent with those that were previously predicted (Fig. 3.1A). These results indicated that transcription of the non-coding DNA strand was at a lower rate. Possibly transcription on this strand was not near a specific start site resulting in less mRNA, which has also been described for genes in *Leishmania major* Friedlin (68). To date, bidirectional transcript has not been described for *Campylobacter* genes. A possible reason for this may be that most researchers only use anti-sense primers for cDNA synthesis or they include both sense and anti-sense primers in the same reverse transcription reaction, which would not show transcription of a single gene in both directions.

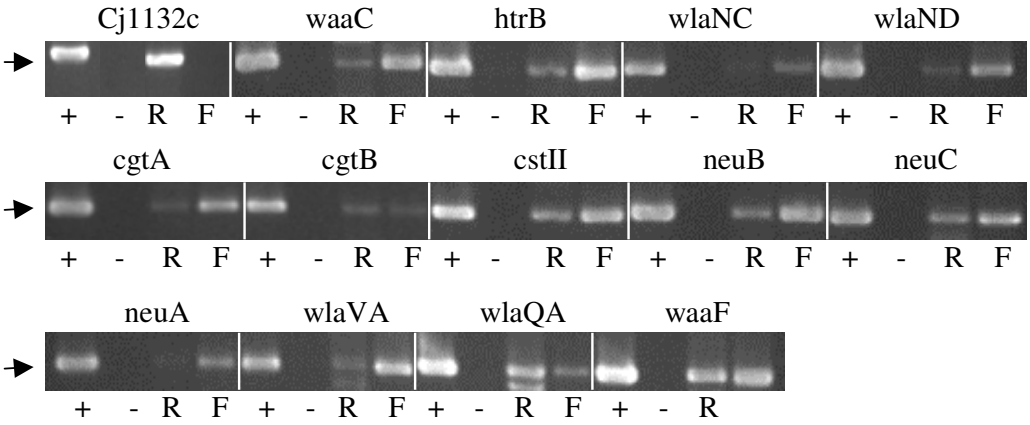

Figure 3.3. Semi-quantitative RT-PCR. Arrows indicate 500 bp position; (+) is positive control; (-) is negative control; (R) is antisense transcript; and (F) is sense transcript.

Table 3.7. Expression of LOS genes under acid stress

Transcript[1]	Mean of C_T value		ΔC_{TC}	ΔC_{TS}	$\Delta\Delta C_T = \Delta C_{TC} - \Delta C_{TS}$	X-fold[4] ($2^{-\Delta\Delta CT}$)
	Normal	Acid				
rpoA-F[2]	24.24	24.00	-	-	-	-
rpoA-R[3]	31.70	31.52	-	-	-	-
htrB-F†	26.06	20.34	–1.82‡	3.66§	–5.48	32
waaC-F	26.05	29.36	–1.81	–5.36	3.55	–12
waaC-R	32.90	40	–1.2	–8.48	7.28	–128
cgtB-F	26.50	40	–2.26	–16.00	13.74	–8192
cgtB-R	21.83	27.07	9.87	4.45	5.42	–32
cstII-F	22.44	29.71	1.8	–5.71	7.51	–128
cstII-R	27.27	40	4.43	–8.48	12.91	–8192
wlaQA-F	29.77	33.76	–5.53	–9.76	4.23	–16
wlaQA-R	23.81	26.71	7.89	4.81	3.08	–8
waaF-F	22.43	25.72	1.81	–1.72	3.53	–12
waaF-R	25.02	30.29	6.68	1.23	5.45	–32

[1], Transcript of genes (for example, rpoA-F and rpoA-R indicate sense and antisense transcript of the *rpoA* gene); [2], internal negative control for sense transcript; [3], internal negative control for antisense transcript; †, internal positive control (only sense transcript was determined); C_T, indicates cycle threshold value; underlined value indicates an estimated value since no product was observed under 40 PCR cycles; ‡, indicates the ΔC_{TC} value of the calibrator calculated by subtracting the C_T value of the rpoA-F under normal conditions from the C_T value of the htrB-F under the same conditions (24.24–26.06 = –1.82); §, indicates the ΔC_{TS} value of the induced sample calculated by subtracting the C_T value of the rpoA-F under acid stress from the C_T value of the htrB-F under the same stress (24.00–20.34 = 3.66); [4], $X > 2$, gene expression is up-regulated; $X < -2$, gene expression is down-regulated.

The role of both sense and antisense transcripts of each LOS gene in responsiveness of *C. jejuni* to acid stress

Prior to starting the gene expression study under acid stress, it was found that *C. jejuni* HB 93-13 showed inhibited growth in brucella broth at pH 5.5 and no growth at pH 5.0. In contrast, other enteric pathogens like *Salmonella typhimurium* were still able to grow in medium at pH 3.0. To survive and cause disease, these bacteria must resist the acidity in the host's stomach. However, this seems most challenging for a fragile bacterium like *C. jejuni*. Therefore, the ability of *C. jejuni* to resist harsh environments in the human gastrointestinal tract is essential for survival, intestinal colonisation, and disease development.

Generally, bidirectional transcription is a very inefficient way to control the gene expression and a waste of resources for the cell as a large portion of each transcript would be unsuitable for translation. To examine physiological significance of bidirectional transcript, differential expression of both sense and antisense transcripts of the *waaC*, *cgtB*, *cstII*, *wlaQA*, and *waaF* genes under acid stress were examined using quantitative real-time RT-PCR. The results showed that when *C. jejuni* HB 93-13 was exposed to acid stress for 15 min, expression of both sense and antisense transcripts of each gene analysed were down regulated (Table 3.7). Therefore, these results showed that both sense and antisense transcripts of the *waaC*, *cgtB*, *cstII*, *wlaQA*, and *waaF* genes play a role in responsiveness of *C. jejuni* to acid stress.

DISCUSSION

To my knowledge, this is the first description of bidirectional transcription of the LOS synthesis gene cluster from *C. jejuni*. The results indicated that both DNA strands of the LOS gene cluster serve as a template for mRNA synthesis, but transcription of the non-coding strands is at a lower rate. Furthermore, the results showed that both sense and antisense transcripts of each LOS gene examined are responsive to acid stress.

Although multiple transcriptional start sites are observed throughout the LOS synthesis gene cluster, recognisable promoter regions are only located upstream of the most upstream initiation sites for the *Cj1132c*, *waaC*, *cgtA*, *cgtB*, *cstII*, *wlaQA*, and *waaF* genes (Table 3.5). Multiples start sites have also been observed for genes in *Leishmania major* Friedlin chromosome 1 (68) and the human type A gamma-aminobutyric acid receptor beta 1 subunit gene (90). In *Neisseria gonorrhoeae*, multiple start sites with multiple promoter sequences were also observed for the LOS-synthesis genes *lgtABCDE*, even for a single *lgtD* gene, and most of these promoters possessed 50 to 67% homology with the consensus gearbox promoter sequence of *E. coli* (12). In *C. jejuni*, an initiation site 91 bp upstream of the start codon of the flagellar biosynthesis *flhB* gene was found using primer extension. However, no recognisable promoter sequence, like a typical σ^{70} or σ^{28} promoter, could be identified immediately upstream of this start site. Additional experiments using transcriptional *flhB*::*lacZ* reporter gene fusions showed that the *flhB* gene did have its own promoter, which was expressed at very low levels (69). Therefore, even though no promoter sequences could be identified in front of some of the initiation sites of the *C. jejuni* LOS genes, these sites could still be functional start sites. However, it could not be excluded that the additional initiation sites were a result of cleavage during RNA processing or RNA preparation, or premature termination of reverse transcription during the 5' RACE procedure. In addition, the identification of multiple start sites might also be a consequence of the lack of regulation of the polymerase enzyme during transcription and/or a very low specificity of the polymerase

(68, 70, 114); as a result, the transcription could initiate indiscriminately at several sites along the LOS gene cluster, and this could result in multiple transcripts for even a single LOS gene.

Three crucial clones carrying gene junction regions in pMW10 could not be obtained. They were the reverse fragments *Cj1132c*/*waaC* and *cgtB*/*cstII* and the same orientation fragment *wlaND*/*cgtA* relative to the *lacZ* reporter gene. Even after multiple attempts no clones of pMW10 with these regions could be obtained. These missing clones might possess functional promoters since (i) multiple start sites on both DNA strands were observed in these regions and (ii) they showed a high similarity of DNA sequences to the *wlaQA*/*waaF* intergenic region, which exhibited bidirectional promoter activity in pMW10 in both forward and reverse directions (Table 3.6, 13.1A and 13.12A).

The cloned *wlaND*/*cgtA* region contains a transcriptional start site with a potential promoter, however no promoter activity was observed. This could possibly be explained if (i) the *cgtA* gene is transcribed using a distal promoter(s) from another gene(s) as previously described for the *C. jejuni fur* gene (105), (ii) the identified start sites would be due to non-specific transcription as a result of low specificity of the polymerase (68, 70, 114), and (iii) this region possesses a very weak promoter, which could not be detected using the *lacZ* reporter gene system.

The consensus sequence of the *C. jejuni* promoter contains three conserved regions, which are located approximately 10, 16, and 35 bp upstream of the transcriptional start site (115). The –10 region consisting of 5'–TATAAT–3', which is very similar to that of a typical σ^{70} promoter of *E. coli* is conserved in *C. jejuni* as is also shown in this study (Table 3.5). However, the –10 region of 5'–TAAAAT–3' is more predominant than that of 5'–TATAAT–3'. The –16 region consisting of 5'–TTTTTTTG–3' is similar to that of a promoter found in Gram-positive bacteria. However, it is unlikely that this conserved region plays an important role, as a large number of homopolymeric tracts and a high A+T content are present in the genome of *C. jejuni* (81). The –35 regions of *C. jejuni* exhibit a high degree of variation and are different

from the –35 region of *E. coli*. This is also observed in this study (Table 3.5). Of these three promoter regions, the –10 region is likely to play the main role in *C. jejuni* promoter activity. Sequence comparison among the strand-switch regions in the genome of *C. jejuni* 11168 (*Cj1009c* to *Cj1289*) (81) revealed a high similarity of DNA sequences for several regions. These included *Cj1132c*/*waaC*, *cgtB*/*cstII*, *wlaQA*/*waaF*, *Cj1137c*/*Cj1138*, *Cj1166c*/*ldh*, *Cj1207c*/*Cj1208*, *Cj1237c*/*pdxJ*, and *gltX2*/*Cj1289*.

There are several possible explanations for bidirectional transcription of the LOS gene cluster. Firstly, there could be initiation sites with bidirectional promoters on both DNA strands in the strand-switch regions. This has also been observed in *Leishmania major* (46, 68) and was also shown in this study for the *Cj1132c*/*waaC*, *cgtB*/*cstII*, and *wlaQA*/*waaF* regions in *C. jejuni*. Secondly, there could be a lack of regulation of the polymerase enzyme during transcription and/or a very low specificity of the polymerase resulting in non-specific transcriptions (68, 70, 114). This could explain why multiple start sites are observed throughout the LOS synthesis gene cluster even for a single gene with and without an active promoter. Thirdly, there might not be a tight control for transcription termination in the LOS gene cluster (*waaC–waaF*). In *E. coli*, it is well known that two major termination mechanisms, Rho-independent and -dependent, are involved in transcription (113). To date, neither of these termination mechanisms have been experimentally demonstrated in the LOS gene cluster of *C. jejuni*. Analysis of the genome of *E. coli* showed that 567 Rho-independent transcription terminators are present (23), while only 9 are present in the genome of *Helicobacter pylori* (23), and 43 in the genome of *C. jejuni* NCTC 11168 (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi). Among these terminators in *C. jejuni*, two are present in the *Cj1117* and *Cj1173* genes, which are located outside the LOS gene cluster (81). This observation indicated that Rho-independent termination most likely does not occur in the LOS gene cluster. This could be supported by our results showing that a transcriptional linkage between operons occurred in the LOS genes (Fig. 3.2). Sturm *et al* have experimentally

shown that transcription can also be terminated by a T tract, which is commonly found in the 3' end of all kinetoplastid SL RNA genes, and that more than six T's are required for efficient termination *in vivo* (98). Possibly, this termination system may also be used by *C. jejuni* since a large number of homopolymeric T tracts are present in the genome of *C. jejuni* (81).

This study also showed the physiological significance of both sense and antisense transcripts of some LOS genes for *C. jejuni* in response to acid stress. Differential expression is observed in the *wla*II-LOS synthesis gene cluster. Under acid stress, the *htrB* expression is up regulated whilst expression of the *waaC*, *cgtB*, *cstII*, *wlaQA*, and *waaF* genes are down regulated. The reduction of expression of the *waaC*, *cgtB*, *cstII*, *wlaQA*, and *waaF* genes suggests a brief growth arrest. This allows the cell to reshuffle energy and to devote this energy to an increased expression of genes involved in a protective response and adaptation to the new growth condition. This reduction reflects the energy-starved condition of the cell and the necessity for saving and reshuffling energy for the increased expression of proteins involved in preventing and repairing damage caused by the acid downshift.

Alain Stintzi previously examined the gene expression profile of *C. jejuni* 11168 under heat stress using microarray (97). Differential expression was observed for several genes in the *wla*I-LOS (*wla-MLKJIHGFEDCB*, *galE*, and *Cj1132c*) synthesis gene cluster (81). Whereas the *wlaE* and *galE* genes were up regulated upon temperature increase, the *wlaK* gene was down regulated. As all genes in the *wla*I-LOS gene cluster are translated in the same direction it would be expected that they would be similarly down or up regulated. However, this discrepancy suggests that the genes from the *wla*I locus are transcribed as multiple operons using different promoters. A similar discrepancy was observed in this study for the *waaC* and *htrB* genes.

It was found that the *htrB* gene is involved in regulating cell responses to various environmental changes including acid, heat, oxidative, and osmotic stresses (Chapter IV). This may be the reason why the *htrB* gene is independently regulated which would explain

why the transcription of its adjacent gene, *waaC*, shows a different response to environmental changes (Table 3.7).

This present study showed bidirectional transcription of the LOS synthesis gene cluster from the GBS-associated *C. jejuni* strain HB 93-13. Both host and bacterial factors are expected to play an important role in GBS development (72, 103, 112). Some *C. jejuni* strains exhibiting human ganglioside-like LOS structures have a potential capacity to induce GBS (117). The LOS synthesis genes, which are essential for the formation of the GBS-inducible determinant, include *galE* (94), *wlaND* (Viraj N. Perera, unpublished data), *cgtA* (35, 37), *cgtB* (62), *cstII* (35), *neuB* (63), *neuC* (35), *neuA* (35), and *waaF* (76). Other bacterial factors, including phase variation (37, 62, 88), natural transformation (86), and bidirectional transcription as described in this study, have a potential effect on the expression of the GBS-inducible determinant. Since expression of some crucial genes *cgtB*, *cstII*, and *waaF* are down regulated under acid stress, further investigation of (i) gene regulation amongst GBS and non-GBS-associated *C. jejuni* strains and (ii) bacterial/environmental factors that up-regulate expression of the GBS-inducible determinant will give a better insight into the pathogenesis of GBS.

CHAPTER IV

Physiological examination of the pleiotropic functions of the LOS-synthesis *htrB* gene of *C. jejuni* HB 93-13

The study described in this chapter was accepted for publication in Microbiology, in press

ABSTRACT

In *Campylobacter jejuni*, a *htrB* homologous gene is located in the lipooligosaccharide synthesis gene cluster. This study examined effects of *htrB* expression in responsiveness of *S. typhimurium* and *C. jejuni* to hash environments. Complementation experiment showed that the *C. jejuni htrB* gene could restore the normal morphology of the *Salmonella htrB* mutant and its ability to grow without inhibition in heat, acid, and osmotic stresses, but not bile stress. This indicated that the *htrB* genes in *C. jejuni* and *S. typhimurium* exhibit similar pleiotropic effects. Moreover, quantitative real-time RT-PCR showed that expression of the *C. jejuni htrB* gene was up regulated under acid, heat, oxidative, and osmotic stresses but it did not change under bile stress. This indicated that the *C. jejuni htrB* gene plays a role in regulating cell responses to various environmental changes. Furthermore, deletion mutation of the *htrB* gene in *C. jejuni* is lethal, indicating that the *htrB* gene is essential for *C. jejuni* survival. Therefore, these results showed that expression of the *htrB* gene is essential for responsiveness of *S. typhimurium* and *C. jejuni* to environmental stresses.

INTRODUCTION

Modulation of lipid A acylation in Gram-negative bacteria results in pleiotropic effects. In *Escherichia coli*, mutation of the *htrB* gene encoding a lipid A acyltransferase leads to inhibition of bacterial growth at high temperature (49, 51), morphological change from short rods to filamentous rods (49), and unusually increased bile resistance (50). In *Salmonella typhimurium*, inactivation of the *htrB* homologous gene exhibits not only the same effects as seen in *E. coli* but also hyperflagellation and severely limited virulence (44, 100). In *Haemophilus influenzae*, knockout of the *htrB* gene resulted in an increased bile sensitivity (58), an increased sensitivity to human antimicrobial peptides (β-defensins) (95), a decreased colonisation capacity (101), a decreased intracellular viability (101), and a decreased pro-inflammatory cytokine induction (104) but it does not affect morphology (58). In *Campylobacter jejuni*, the *htrB* gene is located in the *wla*II-lipooligosaccharide (LOS) synthesis gene cluster and found to be conserved in this bacterium. Functionally, the *C. jejuni htrB* gene encodes a putative acyltransferase involved in lipid A synthesis (29, 32, 81). This study physiologically examined pleiotropic effects as a result of expression of the *htrB* gene of *C. jejuni* strain HB 93-13 using complementation, gene expression, and mutagenesis experiments.

MATERIALS AND METHODS

Bacterial strains and growth conditions

C. jejuni strains HB 93-13 (40), O:4, O:41, O:36, 81116 (78), NCTC 11168, ATCC 43446, and OH 4382 (3) were included in this study. *C. jejuni* was grown on Columbia agar plates supplemented with 5% (v/v) defribinated horse blood under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 42˚C for 16 h unless otherwise stated. *E. coli* DH5α was grown in Luria-Bertani (LB) broth or agar at 37˚C for 16 h. The *S. typhimurium* strains SL1344 (wild type) and the SL1344 *htrB1*::Tn10 (*htrB* mutant, tetracycline resistance) were kindly provided by Dr. Bradley D. Jones, Department of Microbiology, University of Iowa, USA (44, 100). *S. typhimurium* was grown in LB broth or agar at 30˚C for 16 h unless otherwise stated. Media were supplemented with ampicillin (150 μg/ml), μg kanamycin (15 or 50 μg/ml), tetracycline (20 μg/ml), 2% (w/v) X-Gal in dimethyl formamide (40 μl for each LB plate), and 100 mM IPTG (40 μl for each LB plate) when appropriate.

Analysis of DNA and amino acid sequences

Clone manager version 6 (Scientific and Education Software, USA) was used to design primers, plan cloning, and analyse DNA and amino acid sequences. Primers were designed using the *wla*II-LOS synthesis gene cluster of *C. jejuni* strain HB 93-13 (accession no. AY297047) unless otherwise stated.

DNA manipulation

Plasmid DNA was isolated using the mini-prep procedure as previously described (6) and/or using the QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. The DNA quantity was determined using a spectrophotometry. Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from Promega and used according to the manufacturer's instructions. Restriction mapping was performed to confirm the composition and size of the constructed plasmids by digestion with appropriate restriction enzymes.

Transformation of *C. jejuni* with plasmid DNA or genomic DNA was performed using electroporation (25 μF, 1.25 kV, and 600 Ω, gene pulser apparatus, Bio-Rad) and/or natural transformation (biphasic technique) as previously described (108). Transformation of *E. coli* and *S. typhimurium* with plasmid DNA was performed using electroporation (25 μF, 2.48 kV, and 200 Ω, gene pulser apparatus, Bio-Rad). Competent cells for *E. coli* and *S. typhimurium* were prepared in cold 10% (v/v) glycerol as previously described (91).

pfu-PCR

pfu-PCR was used to amplify a DNA fragment from purified chromosomal DNA. The reaction mixture was prepared in a 50 μl total volume of 1 × *pfu* buffer containing 200 μM of each dATP, dTTP, dCTP, dGTP, 100 ng of each primer, 100 ng of DNA, and 5 U of *pfu* polymerase (Roche). The PCR conditions were as follows: 94˚C for 3 min (initial denaturation); 35 cycles of 94˚C for 30 s (denaturation), 50˚C for 1 min (annealing), 72˚C for X min (extension); 72˚C for 7 min (final elongation). X was calculated by dividing the length of the PCR product by 500 bp as *pfu* polymerase synthesizes 500 bp per min.

Colony-PCR

Colony-PCR was used to screen transformants carrying new constructs. The reaction mixture was prepared in a 50 μl total volume of 1 × *Taq* buffer containing 1.5 mM of $MgCl_2$, 200 μM of each dATP, dTTP, dCTP, dGTP, 100 ng of each primer, and 2.5 U of *Taq* polymerase (ABI). A tip of 200 μl in size was used to gently touch a colony on culture plate and directly mixed into the PCR tube containing the master reagent, which had been already prepared. The PCR conditions were as follows: 94˚C for 10 min; 35 cycles of 94˚C for 30 s, 50˚C for 1 min, 72˚C for Y min; 72˚C for 7 min. Y was calculated by dividing the length of the PCR product by 1000 bp as *Taq* polymerase synthesises 1,000 bp per min.

Construction of plasmid carrying the *C. jejuni htrB* gene in the *S. typhimurium htrB* mutant

A 917-bp DNA fragment containing 18 bp of the *waaC* gene (upstream adjacent gene), the entire *htrB* gene (888 bp), and 11 bp of the *wlaNC* gene (downstream adjacent gene) of *C. jejuni* strain HB 93-13 was amplified by *pfu*-PCR with primers *BamH*I-waaC-F (5'-TTGCCAAAGGATCCCTTAATGAAAAATAGTGATAG-3') and *Cla*I-wlaNC-R (5'-TTGTTATCGATTCATTTTGCACCCTTGT-3'). A PCR product was cloned into the pBluescript plasmid in the same orientation as the ampicillin resistance cassette using the *BamH*I and *Cla*I sites, and the resultant construct was subsequently introduced into *E. coli* DH5α by electroporation. Transformants carrying the *htrB* gene constructs were screened using colony-PCR with primers 172-pBlue-F (5'-GGTTCCGATTTAGTGCTTTA-3') and 825-pBu-R (5'-GAAACAGCTATGACCATGAT-3'). These primers were designed to amplify a 1516-bp plasmid fragment, which included a 917-bp inserted PCR product. The pBluescript carrying the *C. jejuni htrB* gene (named pBlue *htrB*+) was isolated from *E. coli* and then introduced into the *S. typhimurium htrB* mutant by electroporation.

Examination of bacterial growth and morphology

The wild type, mutant, and complemented *Salmonella* strains were grown on LB agar plates at 30, 37, and 42˚C for 24 h to an OD_{600} of 0.3. The culture media were supplemented with appropriate antibiotics. Tetracycline was added to growth media used for the *S. typhimurium* mutant, while ampicillin and tetracycline were added into the growth media used for the complemented strain. The ability of bacteria to grow at 30, 37, and 42ºC temperatures was observed. A Gram stain was performed and the morphology was observed under a light microscope.

Examination of bacterial sensitivity to acid and osmotic stresses

To test for acid sensitivity, the wild type, mutant, and complemented *Salmonella* strains (OD_{600} at 0.3) were grown at 30ºC in 0.1% (w/v) peptone water, ranging from pH 2.5 to 7.0 for 24 h. After incubation, the cell density was measured at OD_{600}, and an equal volume of cultured medium was distributed on LB agar plates. All culture media were supplemented with appropriate antibiotics as previously described. The plates were incubated at 30ºC for another 24 h. To test for osmotic sensitivity**,** again the bacteria were grown as previously described, except that 0.1% peptone water (pH 7.0) was supplemented with NaCl (1 to 10% w/v).

Examination of bacterial sensitivity to bile stress

To test for bile sensitivity, the wild type, mutant, and complemented *Salmonella* strains (OD_{600} at 0.3) were grown on LB agar plates containing sodium deoxycholate (DOC; 2, 4, 6, 8, and 10%). The culture media were supplemented with appropriate antibiotics as previously described. The plates were incubated at 30ºC for 24 h.

Treatment of *C. jejuni* with stress environments and RNA preparation

Prior to the treatment of *C. jejuni* with stress environments, the ability of *C. jejuni* HB 93-13 to grow in brucellar broths under heat, acid, osmotic, oxidative, or bile condition was examined. A growth temperature of 44ºC was selected as heat stress since *C. jejuni* was able to grow at 43ºC but not 45ºC. A pH of 5.5 was selected as acid stress since *C. jejuni* showed normal growth at pH 6.0, inhibited growth at pH 5.5, and no growth at pH 5.0. A NaCl concentration of 1.5% was selected as osmotic stress since the bacteria showed normal growth at 1% NaCl, inhibited growth at 1.5% NaCl, and no growth at 2% NaCl. For a *C. jejuni* was unable to grow in normal atmospheric conditions this was used for the oxidative stress challenge. For bile stress, 500 μg/ml of DOC was considered as bile stress (61).

C. jejuni HB 93-13 was grown in 30 ml of brucella broth with gentle shaking under microaerobic conditions at 37ºC for 19 h, and 100 µl of bacterial culture was aliquoted into 6 bottles of brucella broth (30 ml) and incubated for another 19 h. After incubation, 1 ml of brucella broth, which had been supplemented with concentrated HCl, NaCl, or DOC and pre-warmed at 37ºC, was added to culture bottles to obtain a final pH of 5.5 (acid stress), a NaCl concentration of 1.5% (osmotic stress), and a DOC concentration of 500 µg/ml (bile stress). The fourth culture bottle was added with 1 ml of pre-warmed brucella broth and used as the calibrator (normal *htrB* expression level). The fifth culture bottle was immediately moved to the 44ºC incubator (heat stress). The incubation was performed at 37ºC, except the fifth culture bottle, with gentle shaking under microaerobic conditions. The bacteria from the sixth culture bottle was poured onto culture plate and incubated at 37ºC with gentle shaking under normal atmospheric conditions (oxidative stress). Culture samples were collected after incubation for 15 min and 30 min and transferred directly into a 1/10 volume of cold 10 × stop solution [5% (v/v) phenol in 100% ethanol] to halt transcription and RNA degradation. RNA samples were isolated using the RNAgents total RNA isolation system (Promega), and DNA decontamination was performed using the TURBO DNA-*free* Kit (Ambion).

Quantitative real-time RT-PCR

Primers used for cDNA synthesis were as follow. The primers q-htrB-R (5'-TTGAGTGTATTGAGGAAAAC-3'), q-16S rRNA-R (5'-GTATTCTTGGTGATATCTAC-3', accession no. AL111168), q-luxS-R (5'-ATAAATCCTGCGAATAAATG-3', accession no. AL111168), or q-rpoA-R (5'-ATTTGTCCATCAGTTGTTAC-3', accession no. AL111168) were used for synthesis of cDNA for the *htrB*, *16S rRNA*, *luxS*, or *rpoA* genes, respectively.

The cDNA synthesis was performed using the ImProm-II reverse transcriptase (Promega). One microgram of RNA and 50 ng of anti-sense primer in a total volume of 5 µl was heated at 70°C for 5 min and immediately chilled on ice for at least 5 min. The master mix was prepared in a total volume of 15 µl, which consisted of 5 µl of RNase-free water, 4 µl of

ImPorom-II 5 × reaction buffer, 2 μl of $MgCl_2$ (25 mM), 1 μl of dNTP mix (10 mM each dNTP), 2 μl of recombinant RNasin ribonuclease inhibitor (5 u/μl), and 1 μl of Improm-II reverse transcriptase (1 μl/reaction). The master mix was dispensed into the reaction tube containing the mixture of heated RNA and primer. The tube was gently mixed and followed by incubation at 25°C for 5 min, 42°C for 60 min, and 70°C for 15 min. Reverse transcriptase was substituted by RNase-free water for the negative control. After cDNA synthesis, the reaction mixture was added with 10 μl of RNase A solution (20 μg/ml), incubated at 37°C for 20 min, and added with 220 μl of water. For each gene, dilution series of newly synthesised cDNA were made and included in a quantitative PCR to examine the efficiency of PCR.

Primers used for PCR were as follows. The primers q-htrB-F (5'-TTATGCCTGATTGTATCTTG-3') and q-htrB-R as previously described were used to amplify a 125-bp fragment of the *htrB* gene-specific cDNA. The primers q-16S rRNA-F (5'-GTCTCTTGTGAAATCTAATG-3', accession no. AL111168) and q-16S rRNA-R as previously described were used to amplify a 123-bp fragment of the *16S rRNA* gene-specific cDNA. The primers q-luxS-F (5'-AAGTTATGAAAACACCTAAG-3', accession no. AL111168) and q-luxS-R as previously described were used to amplify a 124-bp fragment of the *luxS* gene-specific cDNA. The primers q-rpoA-F (5'-GCTTTAGATGCTTTCTTTAC-3', accession no. AL111168) and q-rpoA-R as previously described were used to amplify a 119-bp fragment of the *rpoA* gene-specific cDNA.

A quantitative PCR was performed on the MyiQ (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The PCR reaction was performed in a total volume of 25 μl, which contained 12.5 μl of iQ SYBR Green Supermix and 12.5 μl of master mix consisting of 1 μl of forward primer (50 ng/μl), 1 μl of reverse primer (50 ng/μl), 3 μl of diluted cDNA, and 7.5 μl of water. Each PCR was performed in duplicate. The same PCR samples that showed variant values of the cycle threshold (C_T) of more than 1 were repeated. PCR conditions were as follows: cycle 1 (1×): 95°C for 5 min; cycle 2 (35×): 95°C

for 30 s, 55 or 60°C for 30 s (55°C for *luxS*; 60°C for *htrB*, *16S rRNA*, and *rpoA*), 72°C for 30 s; cycle 3 (100×): 95°C for 10 s (decrease setpoint temperature after cycle 2 by 0.5ºC, and enable melt curve data collection and analysis). Each specific amplicon was verified both by the presence of a single melting temperature peak and by the presence of a single band of expected size on a 3% agarose gel after electrophoresis. Cycle threshold values were determined with the MyiQ software (Bio-Rad). The relative changes (X-fold) in gene expression between the induced and calibrator samples were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (64). The *16S rRNA*, *rpoA*, and/or *luxS* genes were used as the internal controls. A relative expression value of more than 2 fold was considered significant as up or down regulation.

Construction of the *C. jejuni htrB* mutant

The pBluescript plasmid carrying the mutated *htrB* gene of *C. jejuni* HB 93-13 was constructed. Firstly, a 646-bp DNA fragment containing the partial *waaC* and *htrB* genes of *C. jejuni* HB 93-13 (nucleotide 140-785, AY297047) was amplified by the *pfu*-PCR with primers *EcoR*I-waaC-F1 (5'-ATAGGAATTCATAGCGGTCCAACACA-3') and *BamH*I-htrB-R1 (5'-AATCGGATCCTATTTAGCCGCATAAGC-3'). The PCR product was cloned into the pBluescript plasmid in the forward direction via the *EcoR*I and *BamH*I sites. The resultant construct was subsequently introduced into *E. coli* DH5α by electroporation. A positive clone was selected on LB agar supplemented with ampicillin, X-gal, and IPTG according to the blue and white phenotypes. This procedure identified the pBluescript carrying the partial *waaC* and *htrB* genes (named pBluA). Secondly, the *pfu*-PCR with primers *BamH*I-htrB-F2 (5'-TTACGGATCCAGACTGCGTAGAAAACGA-3') and *Xba*I-wlaNC-R2 (5'-CCTTTCTAGAGATTTTTACGGCTAAGTG-3') was used to amplify a 678-bp DNA fragment containing the partial *htrB* and *wlaNC* genes (nucleotide 934-1611, AY297047). This PCR product was cloned into pBluA in the forward orientation via the *BamH*I and *Xba*I sites. Positive clones were identified by colony hybridisation using the

second PCR product, labelled with digoxigenin using the DIG labelling kit (Roche), as probe. The colony-DNA probe hybridisation was performed at 65˚C overnight, and detection was performed using the alkaline phosphate-conjugated anti-DIG antibody and the NBT/BCIP substrate as described in the user's guide handbook (Roche). Positive clones carrying the *htrB* gene with a 150 bp (nucleotide 785 to 934) deletion were named pBluB. A 1,494-bp kanamycin resistance cassette (Km) was cloned into the pBluB using the *BamH*I site. This resulted in a construct containing part of the *htrB* gene interrupted by the Km (named pBluC) in which the Km was flanked by a 646-bp upstream DNA fragment and a 678-bp downstream DNA fragment. The pBluC carrying the Km in the same direction as the *htrB* gene was named pBluCF, while the construct carrying the Km in the opposite direction to the *htrB* gene was designated pBluCR. Finally, these constructed plasmids were confirmed by sequencing using the ABI sequencing mix V3.1 (ABI) according to the manufacturer's instructions.

Natural transformation and electroporation were used to introduce each recombinant plasmid (pBluCF and pBluCR) into *C. jejuni* strains HB 93-13, O:4, O:41, O:36, 81116, 11168, ATCC 43446, and OH 4382. The pBluescript plasmid carrying the Km within the *wlaVA* gene (pBlu11KR) or the *waaF* gene (pBlu13KF) were used as the positive controls. The pBluescript alone was used as the negative control. Transformants were screened on 5% blood agar plates supplemented with kanamycin (15 μg/ml). Culture media were incubated under microaerobic conditions at 30, 37, and 42˚C for 5 days.

RESULTS AND DISCUSSION

Characteristics of the *C. jejuni htrB* gene

The *htrB* homologue of *C. jejuni* HB 93-13 is located in the *wla*II-LOS synthesis gene cluster. DNA sequence analysis of the *C. jejuni htrB* gene showed an open reading frame of 888 bp initiating with a methionine when translated. Under microaerobic conditions, the *htrB* gene and the other LOS synthesis genes were transcribed as part of several operons using multiple transcriptional start sites with promoters upstream of the start codons of the *Cj1132c*, *waaC*, *cgtA*, *cgtB*, *cstII*, *wlaQA*, and *waaF* genes (Chapter 3). Multiple sequence alignments showed that the *C. jejuni* HB 93-13 HtrB protein was similar to the *E. coli* HtrB (20%, accession no. NC_004431), the *S. typhimurium* HtrB (20%, NC_003197), and the *H. influenzae* HtrB (20%, NC_000907) proteins. Among other bacteria, the *S. typhimurium* HtrB showed 78% similarity to the *E. coli* HtrB and 54% similarity to the *H. influenzae*. These results showed that *S. typhimurium*, *E. coli*, and *H. influenzae* HtrB proteins were closer related to each other than to the *C. jejuni* HtrB protein. In *S. typhimurium*, mutation of the *htrB* gene, which encodes for an acyltransferase enzyme involved in lipid A synthesis, resulted in pleiotropic effects in both pathology and physiology of *S. typhimurium*. These effects included morphological changes from short rods to filamentous rods, hyperflagellation, inability to grow at high temperatures, increased bile resistance, and reduced virulence (44, 100). In this study, the *S. typhimurium htrB* mutant was used as the model for studying pleiotropic effects as a result of expression of the *C. jejuni htrB* gene. The *S. typhimurium htrB* mutant was complemented with the *htrB* gene from *C. jejuni* HB 93-13. The wild type, mutant, and complemented *Salmonella* strains were characterised.

The role of the *C. jejuni htrB* gene in *S. typhimurium* morphology

To examine whether expression of the *C. jejuni htrB* gene affected the morphology of the *S. typhimurium htrB* mutant, the wild type, mutant, and complemented *Salmonella* strains were grown on LB agar plates at 30, 37, and 42ºC for 24 h. The culture media were supplemented

with appropriate antibiotics. A Gram stain was performed and the morphology was observed under a light microscope. The morphology of the complemented and wild type strains was similar showing Gram-negative short rods at 30, 37, and 42ºC, while the morphology of the mutant was Gram-negative filamentous, bulgy, and short rods at all temperatures tested (Fig. 4.1a). These results showed that the *C. jejuni htrB* gene could restore the wild-type morphology of the *S. typhimurium htrB* mutant, and hence its expression affected the bacterial morphology.

The role of the *C. jejuni htrB* gene in growth of *S. typhimurium* at high temperature

To examine whether expression of the *C. jejuni htrB* gene affected the capacity of bacteria to grow at high temperature, the wild type, mutant, and complemented *Salmonella* strains were grown on LB agar plates as previously described. The results showed that the *C. jejuni htrB* gene could restore the ability of the *S. typhimurium htrB* mutant to grow at high temperatures as the complemented and wild type strains grew normally at 30, 37, and 42ºC, while the mutant was temperature sensitive and showed inhibited growth at 37 and 42ºC (Fig. 4.1b). This showed that the *C. jejuni htrB* gene is essential for the *S. typhimurium htrB* mutant to grow properly at high temperature. A previous study showed that the *S. typhimurium htrB* mutant was unable to grow at 37ºC (100) while in this study this bacterial strain showed inhibited growth at 37ºC (Fig. 1b). These inconsistent results could possibly be explained by a loss of temperature sensitivity of the *S. typhimurium htrB* mutant after a few passages at 30ºC, which was also observed for the *H. influenzae htrB* mutant (58).

The role of the *C. jejuni htrB* gene in acid sensitivity of *S. typhimurium*

To examine whether expression of the *C. jejuni htrB* gene affected the acid sensitivity, the wild type, mutant, and complemented *Salmonella* strains were grown at 30ºC in 0.1% (w/v) peptone water (pH 2.5 to 7.0) for 24 hours. After incubation, the cell density was measured at OD_{600}, and an equal volume of culture medium was distributed on LB agar plates. All culture

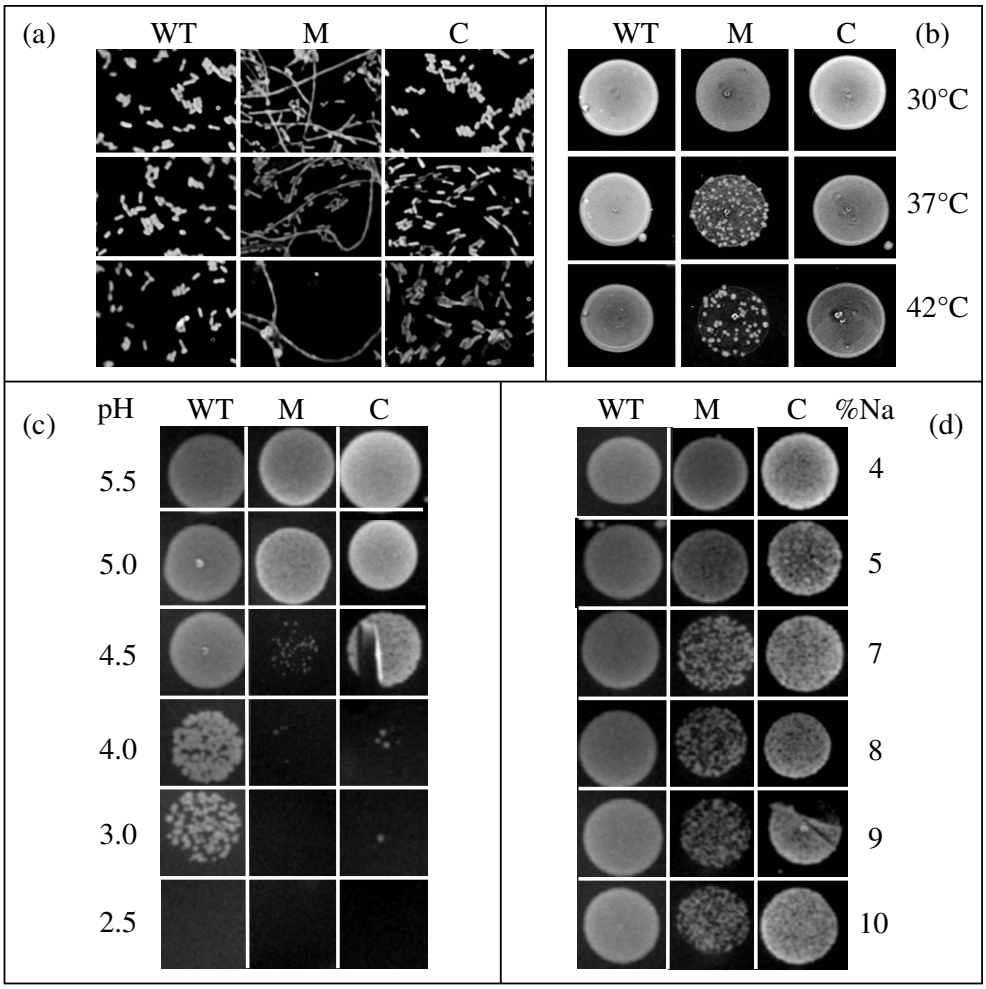

Figure 4.1. Complementation experiments. WT, wild type *S. typhimurium* strain SL1344; M, *S. typhimurium htrB* mutant; C, *S. typhimurium htrB* mutant carrying the functional *htrB* gene from *C. jejuni* HB 93-13; (a), morphology at different temperatures; (b), ability of bacteria to grow at high temperatures; (c), ability of bacteria to grow in high acidity; and (d), ability of bacteria to grow in high osmolality.

media were supplemented with appropriate antibiotics as previously described. The plates were incubated at 30ºC for another 24 h. The results showed that the mutant strain exhibited less growth on LB agar plates compared to the wild type and the complemented strain showed intermediate growth between the wild type and mutant strain at lower pH (Fig. 4.1c, selected results of bacterial growth on LB plates). Therefore, expression of the *C. jejuni htrB* gene contributed to the acid tolerance of the *S. typhimurium htrB* mutant.

The role of the *C. jejuni htrB* gene in high osmotic sensitivity of *S. typhimurium*

To examine whether expression of the *C. jejuni htrB* gene affected the osmotic sensitivity, the wild type, mutant, and complemented *Salmonella* strains were grown as previously described in acid sensitivity assay, except that 0.1% peptone water (pH 7.0) was supplemented with NaCl (1 to 10%). The complemented and wild type strains exhibited a similar growth, while the mutant showed inhibited growth (Fig. 4.1c, selected result). Therefore, the results showed that expression of the *C. jejuni htrB* gene contributed to the osmotic resistance of the *S. typhimurium htrB* mutant.

The role of the *C. jejuni htrB* gene in bile sensitivity of *S. typhimurium*

To examine whether expression of the *C. jejuni htrB* gene affected the bile sensitivity, the wild type, mutant, and complemented *Salmonella* strains were grown on LB agar plate supplemented with DOC concentrations of 2, 4, 6, 8, and 10%. The results showed that the *C. jejuni htrB* gene could not complement the bile sensitivity of the *S. typhimurium htrB* mutant as the mutant and complemented strains were able to grow in all DOC concentrations tested, while the wild type strain was able to grow in DOC up to 8%. Similarly, a bile-resistant phenotype of the *S. typhimurium htrB* mutant was also observed by Sunshine *et al.* (100). However, the study of bile sensitivity in other bacteria showed inconsistent results. The *H. influenzae htrB* mutant was more bile-sensitive than the wild type strain (58), while the *E. coli htrB* mutant was more bile-resistant than the wild type strain (50, 100). Since expression of

the *C. jejuni htrB* gene did not affect the bile sensitivity of the *S. typhimurium htrB* mutant, the *C. jejuni htrB* gene might not have a role in the bile sensitivity in *C. jejuni*.

Expression of the *htrB* gene in *C. jejuni* under stress environments

To examine whether stress environments, including heat, acid, osmotic, oxidative, and bile stresses affected expression of the *htrB* gene in *C. jejuni*, quantitative real-time RT-PCR was performed. Before the quantitative data were accepted, three criteria were required. Firstly, no PCR product should be detected from the negative control without reverse transcriptase, confirming the lack of DNA contamination in the RNA sample. Secondly, PCR efficiency for each gene should be at least 80%. This ensured that low amounts of PCR product were due to low expression levels of the genes of interest, not because of low PCR efficiency. Thirdly, only one peak for each gene analysed should be observed in the melting curve, confirming the lack of non-specific PCR product. In this study, the *rpoA* gene encoding the alpha subunit of RNA polymerase and the *16S rRNA* gene encoding the 16S rRNA subunit (81) were used as the internal negative controls, and it was expected that their expressions should not be affected by change of environments. The *luxS* or *Cj1198* (81), which produces the autoinducer-2 involved in quorum sensing (21), was used as the internal positive control, and its expression should be affected by change of environments. The results showed that there was no ideal and universal internal positive/negative control. For example, after *C. jejuni* was exposed to stress environments for 15 min, expression of the *luxS* gene was not affected under heat and bile stresses but it was affected under other environments. Expression of the *16S rRNA* gene showed a lesser variation under osmotic and oxidative stresses but it showed a higher variation under other environments when compared to other genes (Table 4.1). As a result, for each environment, expression of the control gene that showed lowest variation was selected as the internal control. Therefore, at 15 min, the *rpoA* gene was selected as the internal control for studying the *htrB* expression under acid stress, the *luxS* gene was used as the control under heat and bile stresses, and the *16S rRNA* gene was selected as the control

Table 4.1. Expression of the *C. jejuni htrB* gene under stress environments

Condition	C_T of genes				ΔC_T calibrator[a]	ΔC_T htrB[b]	$\Delta\Delta C_T$[c]	$2^{-\Delta\Delta CT}$ [d]
	htrB	16S	luxS	rpoA				
At 15 min								
Normal	26.06	11.33	31.63	24.24				
Acid	20.34	10.58	29.6	<u>24.00</u>	–1.82	3.66	–5.48	44.63
Bile	25.53	7.88	<u>31.29</u>	ND	5.57	5.76	–0.19	1.14
Osmotic	23.32	<u>9.83</u>	29.44	ND	–14.73	–13.49	–1.24	2.36
Heat	23.32	8.58	<u>31.13</u>	ND	5.57	7.81	–2.24	4.72
Oxidative	20.35	<u>8.37</u>	25.94	19.51	–14.73	–11.98	–2.75	6.73
At 30 min								
Normal	20.49	10.31	28.10	21.07				
Acid	18.39	9.68	<u>28.59</u>	ND	7.61	10.20	–2.59	6.02
Bile	20.32	<u>9.81</u>	29.98	ND	–10.18	–10.51	0.33	–1.26
Osmotic	21.02	<u>10.03</u>	26.38	ND	–10.18	–10.99	0.81	–1.75
Heat	20.75	8.60	29.39	<u>21.70</u>	0.58	0.95	–0.37	1.29
Oxidative	19.03	<u>10.12</u>	26.03	20.81	–10.18	–8.91	–1.27	2.4

ND, not determined; C_T, cycle threshold value; underline, C_T value of the internal control genes that did not change or slightly affected by change of environments, these genes were selected as the internal controls under particular environments. For example, at 15 min, the *rpoA* gene was used as the control gene under acid stress as its expression (C_T) was not affected by acid stress (24.00 versus 24.24 under normal conditions.

[a], ΔC_T calibrator was calculated by subtracting the C_T value of the control gene under normal conditions from the C_T value of the *htrB* gene under normal conditions.

[b], ΔC_T htrB was calculated by subtracting the C_T value of the control gene under stress from the C_T value of the *htrB* gene under the same stress.

[c], $\Delta\Delta C_T$ was calculated by subtracting the value of the ΔC_T calibrator from the value of the ΔC_T *htrB*.

[d], $2^{-\Delta\Delta CT} > 2$, gene expression was up regulated; $2^{-\Delta\Delta CT} < -2$, gene expression was down regulated; $-2 \geq 2^{-\Delta\Delta CT} \leq 2$, gene expression was not affected by change of environments.

under oxidative and osmotic stresses. At 30 min, the *luxS* and *rpoA* genes were selected as the control genes under acid and heat stresses, respectively. The *16S rRNA* gene was selected as the control gene under oxidative, bile, and osmotic stresses. After the expression level of the *hrtB* gene was subtracted with that of the internal control, it was found that after *C. jejuni* was exposed to each artificial environment for 15 min, expression of the *htrB* gene was highly up regulated under acid stress, moderately up regulated under heat and oxidative stresses, slightly up regulated under osmotic stress, and did not change under bile stress. After 15 min of exposure, its expression gradually decreased with time to reach/close to a baseline level at 30 min. At this time point, expression of the *htrB* gene was moderately up regulated under acid stress, slightly up regulated under oxidative stress, and did not change under bile, osmotic, and heat stresses (Table 4.1). These results showed that the *C. jejuni htrB* gene is involved in regulating cell responses to various environmental changes. This is consistent with our previous results that the *C. jejuni htrB* gene is essential for the *S. typhimurium htrB* mutant to grow at high temperatures, acidity, and osmolality.

The results of the real-time RT-PCR experiments showed that expression of the *C. jejuni htrB* gene was up regulated under harsh environments, including heat stress. In contrast, a previous study showed that expression of the *E. coli htrB* gene was not affected by heat-shock using Northern blot analysis (51). It was proposed that the *E. coli htrB* gene is a member of a new class of genes whose products are required for growth at high temperature but are not heat shock genes (50). Similarly, expression of the *htrB* gene of *C. jejuni* NCTC 11168 was not significantly up or down regulated after the growth temperature had been shifted from 37 to 42ºC using microarray analysis (97). The cause for this inconsistent result might be due to the sensitivity of the methods used. According to the results presented in this study and the fact that real-time RT-PCR is the most sensitive method for differential gene expression, therefore expression of the *C. jejuni htrB* gene is affected by heat-shock.

Effect of modulation of lipid A acylation in *C. jejuni*

To examine the effect of modification of lipid A acylation in *C. jejuni*, the acyltransferase-encoding *htrB* homologous gene was inactivated using mutagenesis experiments. Firstly, two recombinant pBluescript plasmids were constructed. The first construct carried the Km within the *htrB* gene in the same orientation (pBluCF). The second construct carried the Km in the reverse orientation to the *htrB* gene (pBluCR). Secondly, natural transformation and electro-transformation were used to transform various *C. jejuni* strains with these constructs. Plasmids carrying a Km within the *wlaVA* gene (pBlu11KR) and within the *waaF* gene (pBlu13KF) were used as the positive controls. The pBluescript without insert was used as the negative control. Transformants were screened on a selective media that was supplemented with a low concentration of kanamycin (15 μg/ml) since the *H. influenzae htrB* mutant was previously shown to be hypersensitive to kanamycin (58). The culture media were incubated at 30, 37, and 42ºC for 5 days since a previous study showed that the *E. coli htrB* mutants could not initially grow on rich media at temperatures above 33ºC (49). As expected, a number of *C. jejuni waaF* and *wlaVA* mutants were obtained from the positive controls, and no transformants were recovered from the negative control. This showed that the possibility that different restriction modification systems between *C. jejuni* and *E. coli* would be a barrier for interstrain plasmid transfer could be ruled out. No transformants carrying the mutated *htrB* gene as a result of homologous recombination via a double cross over event were recovered on the selective medium. However, a few transformants carrying both an intact and a mutated *htrB* gene resulting from a single cross-over were observed. After several passages of these transformants and after transformant-derived genomic DNA was introduced into the parental *C. jejuni* strain HB 93-13, individual progenies still carried both mutated and intact *htrB* genes (results not shown). These results indicated that the *htrB* gene is essential for *C. jejuni* survival and hence gene deletion in *C. jejuni* causes loss of cell viability.

A previous study showed that the plasmid transformation frequency via homologous recombination directly correlates with the size of the flanking regions (108). DNA recombination between the inserts present in suicide vectors and the genome occurred with as little as 200 homologous base-pairs present (108). The plasmids pBluCF and pBluCR carried the Km flanked by a 646-bp upstream region consisting of the partial *waaC* and *htrB* genes and a 678-bp downstream region consisting of the partial *htrB* and *wlaNC* genes. Therefore the sizes of these flanking regions should have been sufficient to initiate homologous recombination in *C. jejuni*.

The possible explanations why the *htrB* gene could be mutated in other bacteria but not in *C. jejuni* are as follows. Firstly, unlike most other bacteria *C. jejuni* is a fragile enteric bacterium. For example, it was found that the growth of *C. jejuni* was not observed in brucella broth with NaCl > 1.5%, DOC > 2% or pH < 5.5. In addition, *C. jejuni* is hypersensitive to normal atmospheric conditions. In contrast, the growth of *S. typhimurium* was still observed in a medium consisting of 10% NaCl, 10% DOC, or pH 3.0, and it was able to grow under normal atmospheric consitions. Secondly, the results of the complementation and gene expression presented here indicated that the *C. jejuni htrB* gene is involved in morphology (cell wall formation) and is essential for growth under stress environments, including acid, heat, osmotic, and oxidative stresses. Thirdly, accomplishment of inactivation of the *htrB* genes might depend on the mutagenesis-based techniques. In this study, deletion mutation was approached to inactivate the *htrB* gene in *C. jejuni* (see method). Several attempts were made without success. In contrast, insertional mutagenesis of the *htrB* genes using transposon-based technique was successful in *S. typhimurium* (100), *E. coli* (49), and *H. influenzae* (58). Similarly, deletion mutation of the *waaC* gene, which is located upstream of the *htrB* gene, resulted in loss of viability of *C. jejuni* strain 81116 (Benjamin N. Fry, unpublished data), while insertion mutation of the *waaC* homologous gene in *C. jejuni* strain 81-176 was

successful (45). Therefore, deletion mutation of the *C. jejuni htrB* gene might result in cell wall dysfunction, such as loss of membrane permeability, leading to bacterial cell death.

The role of the *C. jejuni htrB* gene in lipid A synthesis is controversial. A number of previous studies have proposed that the *C. jejuni htrB* gene encodes a putative acyltransferase involved in lipid A synthesis (32, 81). Since bile is responsible for digesting fats by disaggregating the lipid bilayer of the cellular membrane, inactivation of the lipid A-synthesis gene should increase the bile sensitivity of bacterial cells. Generally, if the *C. jejuni htrB* gene has a role in lipid A synthesis, its expression should be affected under bile stress. This study indicated that the *htrB* gene might not play a role in lipid A synthesis since its expression did not change when *C. jejuni* was exposed to bile stress. Additionally, it could not complement the bile sensitivity of the *S. typhimurium htrB* mutant. As the role of the *S. typhimurium htrB* gene in lipid A synthesis could be demonstrated by mass spectrometric analysis of the crude lipid A fraction, this technology could possibly also be used to analyse the *C. jejuni htrB* gene and its role in lipid A synthesis.

CONCLUSION

This study showed that (i) expression of the *C. jejuni htrB* gene is essential for responsiveness of *S. typhimurium* to stress environments, (ii) the *C. jejuni htrB* gene involves in regulating cell responses to environmental changes, and (iii) it is likely that the *htrB* gene is essential for *C. jejuni* survival.

GENERAL DISCUSSION AND FUTURE DIRECTION

In this study, insights into the lipooligosaccharide (LOS) synthesis genes in relation to pathophysiology of *C. jejuni* were made. These included mechanisms contributing to diversity of LOS genes (Chapter I), physiological properties of DNA/RNA (Chapter II), unusual transcription of LOS genes (Chapter III), and pleiotropic effects of the LOS molecule (Chapter IV).

Polymorphism is considered as a major trait of *C. jejuni*. This genetic variation provides a population with genome plasticity that contributes to the adaptation and survival of this bacterium in hostile environments. Molecular epidemiological analysis suggests that *C. jejuni* has a largely non-clonal population structure, and genotype diversity is generated continuously (22). A number of genetic mechanisms have been proposed which contribute to the genetic diversity including horizontal transfer of genes within and between bacterial populations (Chapter I) (7, 11, 30, 86) and intragenomic events such as rearrangements, point mutations, deletions, duplications, inversions, and phase variation (1, 32, 37, 62, 88, 110). These events explain the genome plasticity, including the diversity of the LOS synthesis gene clusters, in *C. jejuni*.

Instability of the *C. jejuni* LOS gene cluster limits current typing systems. Currently, there are two LOS-based typing systems, the Penner serotyping system (85) and the LG typing system (92). The Penner system is based on antibodies to LOS and/or capsule (48), while the LG typing system is based on PCR-RFLP patterns of the *wla*I-LOS synthesis gene cluster. Generally, it is assumed that typing systems should be based on traits that exhibit diversity and stability. In *C. jejuni*, the LOS structures reveal diversity among strains as a result of horizontal gene transfer within and between strains (Chapter I) (7, 11, 30, 86) and phase variation (37, 62, 88). However, the LOS structure is not stable as certain strains, like *C. jejuni* 81116, can change their entire LOS gene clusters as well as their LOS structures following natural transformation (Chapter I). Therefore, LOS based typing systems are not

ideal and the search for better traits to be used for typing of *C. jejuni* strains should be continued.

***C. jejuni* DNA or RNA has different physiological properties to that of other bacteria.** While attempting to analyse transcription of the LOS synthesis gene cluster, it was observed that obtaining a DNA-free RNA sample from *C. jejuni* proved difficult. Many RNA methods were tried on their own or in combination and a DNA-free RNA sample could only be obtained using a combination of RNAzolB, TURBO DNAse treatment, and acid phenol extraction. The removal of contaminating DNA from an RNA sample seemed especially difficult for RNA isolated from *C. jejuni*. For example, 10 μg of genomic DNA isolated from *E. coli* DH5α could be completely eliminated by treatment with DNase for 1 hour. In contrast, DNase treatment of a *C. jejuni* RNA sample was not successful although the same RNA sample was repeatedly treated with DNase. This observation indicated the possibility that (i) bacterial factors, such as the methylation system, contribute to the tolerance of *C. jejuni* DNA to DNase, and (ii) DNase enzymes used in this study possess a low specificity to *C. jejuni* DNA. Thus, further investigations of (i) the association between the methylation system and expression of the LOS synthesis genes and (ii) a comparative functional study between *C. jejuni* DNase and other bacterial DNases may unravel the mechanism behind this phenomenon. Moreover, two patterns of total RNA were observed for different *C. jejuni* strains. Some strains exhibited a normal RNA pattern showing three separate bands, corresponding to 23S rRNA, 16S rRNA, and 5S rRNA, as found in other Gram-negative bacteria. In contrast, the RNA pattern of most *C. jejuni* strains tested revealed four bands. It seems likely that the 23S rRNA fragment is cleaved resulting in two molecules, one larger and one smaller than the 16S rRNA. Again, further characterisation of the 23S rRNA genes/proteins may unravel its particular role in relation to the pathophysiology of *C. jejuni*.

The LOS molecule of *C. jejuni* exhibits pleiotropic effects. The LOS is a major surface component, which is divided into two major parts, the lipid A and the core oligosaccharide

consisting of the inner core and the outer core. The core oligosaccharide contributes to the virulence of *C. jejuni* (24) and the induction of GBS (117). The lipid A of the LOS molecule possesses endotoxic properties (73). Expression of some of the LOS synthesis genes also results in multiple effects. For example, expression of the *waaC* gene affects both the LOS-inner core synthesis and the capsule synthesis (45), while expression of the *neuB* genes affects both the LOS-outer core synthesis and the flagella synthesis (63). Another example is the *htrB* gene. It is involved in morphology, viability, growth capacity, and sensitivity to stress environments (Chapter IV). Based on this data, it will be clinically useful for further functional characterisation of the HtrB homologous proteins in other enteropathogens as these proteins may represent a new antimicrobial target(s) for chemotherapy.

Both bacterial and host factors play an important role in GBS development. GBS is an autoimmune neuropathy, which can occur following a *C. jejuni* infection. It is known that some *C. jejuni* strains exhibiting human ganglioside-like LOS structures can induce GBS (117). The LOS synthesis genes, which are essential for the formation of this GBS-inducible determinant, include *galE* (94), *wlaND* (Viraj N. Perera, unpublished data, school of Applied Sciences, RMIT University, Australia), *cgtA* (35, 37), *cgtB* (62), *cstII* (35), *neuB* (63), *neuC* (35), *neuA* (35), and *waaF* (76). Other bacterial factors, including phase variation (37, 62, 88), natural transformation (Chapter I) have a potential effect on the expression of the GBS-inducible determinant. Surprisingly, both DNA strands of the LOS synthesis gene cluster of GBS-inducing *C. jejuni* HB 93-13 are transcribed but transcription of the non-coding strands is at a lower rate, and both sense and antisense transcripts of each LOS gene tested are involved in regulating *C. jejuni* responses to acid stress (Chapter III). This unusual transcription might also be involved in the expression of the GBS-inducing determinant. A role of host factors in relation to GBS is implied by the fact that (i) a cross-reactive immune response to the *C. jejuni* LOS and the human gangliosides is rare following a *C. jejuni* infection (72, 103) and (ii) the same *C. jejuni* isolates are observed from both GBS and non-

GBS patients (112). Although a number of investigators have been trying to link the host factors like HLA, the T cell receptor, and the LPS/LOS receptor (CD14 and TLR4) to GBS, direct evidence of these host factors in relation to GBS development has not been established and needs future research (27, 28, 38, 39, 60, 65, 66, 71, 89).

CD14 and Toll-like receptor 4 (TLR4) are important in the presentation and intracellular signalling of LPS. CD14 is a 55-kDa glycoprotein, which is mainly expressed on mature monocytes, macrophages, and activated granulocytes (118). TLR4 is a transmembrane protein required for intracellular signalling. After the LPS molecule binds to its receptor, the CD14 and TLR4 complex, a cascade of intracellular events results in the activation of NF-κB, which in turn induces the transcription of several cytokine genes such as IL-1, IL-6, and TNF-α resulting in a pro-inflammatory environment (55). In the case of GBS, the induction of cross-reactive antibodies to the *C. jejuni* LOS and the human gangliosides may be caused by inappropriate activation of antigen presenting cells *via* the CD14-TLR4 complex. However, polymorphisms in LOS receptors CD14 and TLR4 do not seem to be associated with disease susceptibility or *C. jejuni* infection in GBS patients (27). Therefore, other host factors involved in LOS presentation through the CD14-TLR4 complex, such as LOS-binding proteins, should be the focus of future investigations.

Investigation of LOS-associated host proteins may unravel the pathogenesis of GBS. Three host proteins are well known to play an important role in host immune responses to the LPS of Gram-negative bacteria. These proteins are the 18-kDa cationic antimicrobial protein (CAP18, LL-37), the bactericidal permeability-increasing protein (BPI, CAP57), and the LPS binding protein (LBP). CAP18 is a human antimicrobial peptide released from activated neutrophil granulocytes which binds to LPS and neutralises its various effects (57). BPI is a human antimicrobial peptide found in granulocytes and bone marrow-derived dendritic cells (19, 67). It binds to LPS near the lipid A domain, and formation of the LPS-BPI complex abrogates detrimental host responses to LPS (67). The mouse BPI is most strongly induced by

bacterial LPS through a signaling pathway that is completely dependent on its TLR4/IL-1 receptor domain which induces the synthesis and secretion of IFN-β (19). Relation to GBS, the presence of BPI could prove valuable in prognosis as it will result in a reduced LOS responsiveness. LBP is an acute-phase protein that is mainly synthesised in hepatocytes and is released as a glycoprotein into the bloodstream. Other sources of LBP include epithelial cells, renal cells, and the central nervous system. At lower concentrations, LBP binds and transfers LPS to both the membrane bound (CD14-TLR4 complex) and the soluble form of CD14, resulting in the activation of host immune responses to the LPS (120). However, concentrations of LBP in human serum increase during an acute-phase response, and these increased LBP concentrations exhibit inhibitory effects in terms of cellular activation. Therefore, investigating the presence or absence of the LPS/LOS-binding proteins in GBS patients might lead to a better understanding of the development of GBS. Furthermore, comparative analysis of the genomes/proteomes of hepatocytes and peripheral blood mononuclear cells isolated from both GBS and non-GBS patients might lead to the identification of the host susceptibility genes/proteins.

Vaccine development against *C. jejuni* faces serious challenges. To date, no effective vaccine is available. From the data presented in this thesis it is apparent that LOS should be used with caution as antibodies to LOS of some *C. jejuni* strains have been shown to cross-react with human neuronal gangliosides (117). Furthermore, a live vaccine of *C. jejuni* should not be used as certain *C. jejuni* strains, such as *C. jejuni* 81116, can change both phenotype and genotype following natural transformation (Chapter I). In addition, this strain can transform from a non-GM1-expressing strain to a number of GM1-expressing strains (Chapter I), which have the potential to induce GBS (117). However, a vaccine containing killed bacteria lacking ganglioside-like LOS structures could be considered. Since *C. jejuni* exhibit genetic diversity among strains, immunity to *Campylobacter* appears to be strain-specific and not protective (10). Therefore, subunit vaccines seem to be more appropriate for this bacterial

pathogen. Antigenic determinants that are essential for survival, growth, resistance to stress environments, and/or virulence, such as the HtrB protein (Chapter IV) should be considered as candidates to be included as a subunit vaccine.

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